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# ABSTRACT

Serum levels of prostate specific antigen (PSA) serve as the current mainstay in early prostate cancer (PCa) detection. However, due to the unspecificity of PSA, additional diagnostic measures are needed, urging the development of better markers. Recent studies indicate that urine samples, which contain prostate specific proteins and nucleic acids, are a potential biomarker reservoir. The aim of this study was to detect and analyze urinary RNA markers that may provide a novel non-invasive way for PCa diagnosis.

Urine samples collected after routine digital rectal examination were obtained from 30 benign patients (BE: elevated serum PSA, tumor-negative prostate biopsy), 20 low grade PCa patients (LG: positive biopsy, Gleason score ≤7 in radical prostatectomy specimen); 10 high grade PCa patients (HG: positive biopsy, Gleason score ≥7 in radical prostatectomy specimen). Total RNA was isolated from exosomes prepared by ultrafiltration of cleared urine samples using the tri-reagent. RNA was reverse-transcribed to cDNA and subsequently pre-amplified using a pool of 33 primer pairs. Finally, quantitative real time PCR was performed for a panel of known and novel candidate PCa mRNA markers, micro-RNAs, and transcript variants. Statistical significance was determined via One-Way ANOVA, Mann-Whitney U-test and corrected by multiple testing.

A panel of five genes (RHOA, HPRT1, TBP, PA2G4 and ACTB) was tested for their usability as housekeeping genes (HKGs). From those only RHOA showed typically consistent expression in benign and cancer groups and therefore has been taken for normalization of the expression of other genes. The data analysis of 30 genes showed quantitative differences in expression. Prostate specific transcripts (PSA, KLK2, PCA3) were detected in 94 % of benign samples and in 100% of the cancer samples. More than 15 genes were identified to be differentially expressed in a significant way between subgroups. TFF3, PCA3, ACTB, PA2G4, PSA, KLK2, VDAC1 have been identified as the best discriminatory RNA marker candidates in urinary exosomes.

Our pilot study for the measurement of PCa RNA markers in urine derived exosomes supports the idea that urine exosomes may be a future sample source for PCa diagnosis, which needs further optimization of the analysis.

# KURZFASSUNG

Serumspiegel des Prostata-spezifischen Antigens (PSA) dienen als aktueller Standardmarker in der Früherkennung von Prostatakrebs (PCa). Aufgrund des unspezifischen Charakters von PSA sind jedoch zusätzliche diagnostische Maßnahmen erforderlich. Neue Studien weisen darauf hin, dass Urinproben, die Prostata-spezifische Proteine und Nukleinsäuren enthalten, potentielle Träger eines Reservoirs von PCa Biomarkern sind. Ziel dieser Studie ist es, neue, nicht-invasive Urin RNA-Marker zu detektieren und analysieren, welche PCa Diagnosen unterstützen.

Urinproben von 30 gutartigen Patienten (BE: erhöhtes Serum-PSA, Tumor mit negativer Prostata Biopsie), 20 “low gleason“ PCa-Patienten (LG: positive Biopsie mit Gleason Score ≤7 in radikalen Prostatektomie Proben) und 10 “high gleason“ PCa-Patienten (HG: positive Biopsie mit Gleason score ≥7 in radikalen Prostatektomie Proben) wurden nach der routinemäßigen digitalen rektalen Untersuchung gesammelt. Die gesamt-RNA wurde aus Exosomen mittels Tri-Reagenz via Ultrafiltration isoliert. Anschließend wurde die isolierte RNA in cDNA via Reverse Transkriptase PCR umgeschrieben und unter Verwendung 33 spezifischer Primerpaare vervielfältigt. Abschließend wurde eine quantitative real-time PCR mit einer Gruppe von bekannten neuen PCa mRNA / micro-RNA-Markern und Transkriptvarianten durchgeführt. Die statistischen Signifikanzen wurden via One-Way ANOVA mittels SPSS Software bestimmt.

Eine Gruppe von fünf Genen (RHOA, HPRT1, TBP, PA2G4 und ACTB) wurde für die Verwendbarkeit als Housekeeping-Gene (HKGs) getestet. Da nur RHOA eine typische konsistente Expression in der benignen Gruppe als auch in den malignen Gruppen zeigte, wurde es für die Normalisierung ausgewählt. Die Analyse von 30 Genen zeigte quantitative Unterschiede in der Expression. Prostata-spezifische Transkripte (PSA, KLK2, PCA3) wurden in 94% der benignen und in 100% der malignen Proben nachgewiesen. Mehr als 15 Gene wurden zwischen den Untergruppen als signifikant identifiziert. Weiters wurden TFF3, PCA3, ACTB, PA2G4, PSA, KLK2 und VDAC1 als die besten diskriminierenden RNA Marker-Kandidaten aus Urin Exosomen identifiziert.

Diese Studie liefert einen Machbarkeitsnachweis für die Bestimmung von PCa Markern aus Urin Exosomen und zeigt deren mögliche Rolle als Probenquelle in der Zukunft der PCa Diagnose.

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# 1 INTRODUCTION

## 1.1 The Prostate

### 1.1.1 Embryology, Anatomy and Function of the Prostate Gland

#### *Embryologic Origin*

The development of the prostate starts at the 12th week of embryonic life under the influence of androgenic hormones from the fetal testes. During the first 10 weeks of gestation, testosterone from the embryonic testes stimulates the ingrowth of epithelial buds into the urogenital sinus mesenchyme through a feedback loop. Its basic structure is formed between week 31 and 36 of gestation. Up to the puberty the prostate grows slowly. During puberty androgen secretion induces prostate growth up to a weight of averagely 20g in 20 year old males. Its shape equals an inverted cone, with the base at the bladder neck and the apex at the urogenital diaphragm (1). During the third decade of life the glandular epithelium grows into the lumen of the follicles by irregular multiplication of the epithelial infoldings. After the third decade the size remains unaltered until an age of 45 to 50, where the prostate may undergo benign hypertrophy or alternatively progressive atrophy (2).

#### *Anatomy and physiology*

The size of the healthy adult prostate is similar to a chestnut. It is conical in shape and its volume is about 20 to 30ml. With increasing age it may enlarge up to five or six times in size. As can be seen in Figure 1, the prostate is situated in the true pelvis, behind the inferior border of the symphysis pubis and the pubic arch lying in front of the ampulla of the rectum, through which it may be palpated digitally. The prostate is also surrounded by another fascia called the prostatic sheath, which primarily is made up of fibrous tissue in which a rich plexus of veins is inserted (3). The prostatic urethra does not follow a straight line as it runs through the center of the prostate gland. It is actually bent anteriorly 35 degrees at the verumontanum (where the ejaculatory duct joins the prostate) (4).

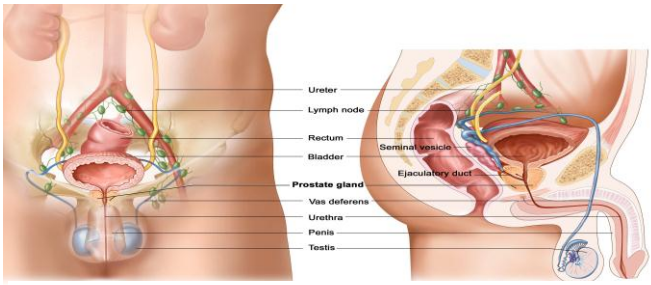



**Figure 1** Prostate gland with beside structure.

(http://www.cancer.gov/PublishedContent/MediaLinks/626489.html, 04.07.2012)

Since a prostate is a gland, it produces and secretes a thin, milky slightly alkaline fluid that contains calcium, citrate, phosphate, zinc and fibrolysin that nourishes and protects sperm and controls the flow of fluid. The prostate specific antigen (PSA) is a constituent found in prostatic secretions. The cells lining the prostate gland produce components of the semen that exits the penis at the time of sexual climax (orgasm). By neutralizing the acidic environment in the female reproductive tract, prostatic fluid enables optimal conditions for sperm by increasing mobility and fertilization of the ovum.

### *Zonal anatomy of the prostate*

According to McNeal's model (see Figure 2), the prostate is composed of four different anatomical zones that have anatomical-clinical correlations (4).

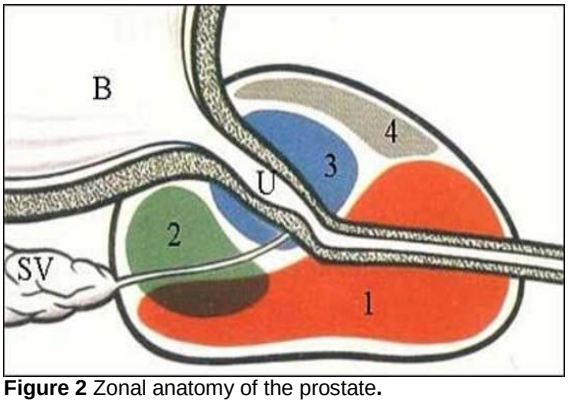



**Figure 2** Zonal anatomy of the prostate**.**
1= Peripheral Zone, 2= Central Zone, 3= Transitional Zone, 4= Anterior Fibromuscular Zone. B= Bladder, U= Urethra, SV= Seminal Vesicle (Algaba F. Lobar division of the prostate) (5).

**1)** The peripheral zone is the area forming the posteria-inferior part of the gland and represents 70% of the prostatic volume. It is the most common site (60-70%) where prostate cancer (PCa) occurs. **2)** The central zone constitutes 25% of the prostate volume and contains the ejaculatory ducts. It usually gives rise to inflammatory processes like prostatitis. **3)** The remainding 5 % of the gland are represented by the transitional zone, where benign prostatic hypertrophy frequently occurs. It consists of two lateral lobes together with periurethral glands. Approximately 25% of prostatic adenocarcinomas occur in its transitional zone. **4)** The anterior zone predominantly fibromuscular with no glandular structures.

### *1.1.2 Benign prostate diseases*

The pathological/abnormal enlargement of the prostate gland can be caused by different reasons:

The **Prostatitis**, a bacterial inflammation or infection of the prostate gland, is characterized by discomfort, pelvic pain, irritated voiding symptoms, fever and sexual dysfunction. It is the most common prostate disease in men younger than 50 years.

**Benign prostatic hyperplasia (BPH)** is the most frequent non-cancerous cause of prostate enlargement in men aged 60 or older (6). BPH causes urination difficulties and occasionally urine retention (Prostatism) by constricting the urethra (Figure 3). According to Ourad *et al* it is manifested by an obstruction or irritation related to overactivity of the bladder (7).

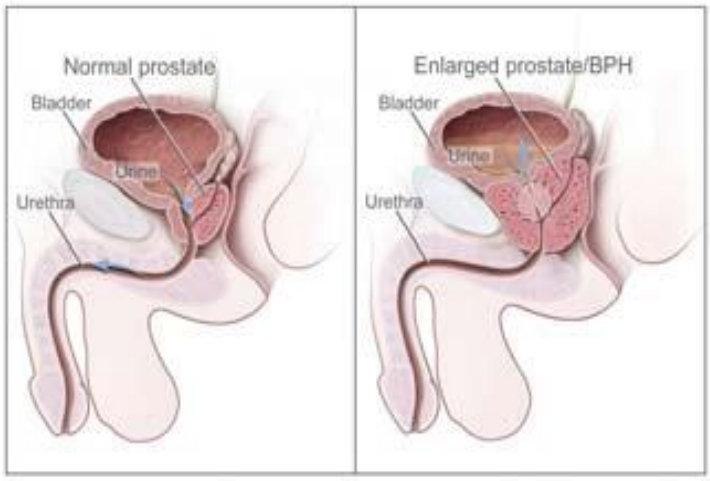



**Figure 3** Benign prostatic hyperplasia (BPH).
The healthy prostate does not inhibit urine flow (left). In BPH (right) the urethra is constricted by the enlarged prostate causing an increase in urine retention. (http://www.cancer.gov/PublishedContent/MediaLinks/626490.html, 08.07.2012)

## 1.2 Prostate Cancer

### 1.2.1 Epidemiology, Incidence and Risk factors

Prostate cancer (PCa) is the second most frequently diagnosed cancer in European men (8) and the sixth most common cancer worldwide. PCa cells occur in approximately half of all men in their fifties. Moreover, small cancerous areas are found retained in 8 out of 10 men aged over 80 years. PCa incidence varies by race and ethnicity. Early studies have shown that African-American men have statistically the highest incidence of PCa. In 2008, 899,000 men were diagnosed with PCa worldwide, whereas more than two-third of cases were diagnosed in developed countries (Figure 4) (9).

On the contrary, PCa incidence in African and Asian men is relatively lower. This difference may be explained by frequently applied PSA screenings in the Western countries (10), cultural variations in nutrition and/or genetic factors (11, 12).

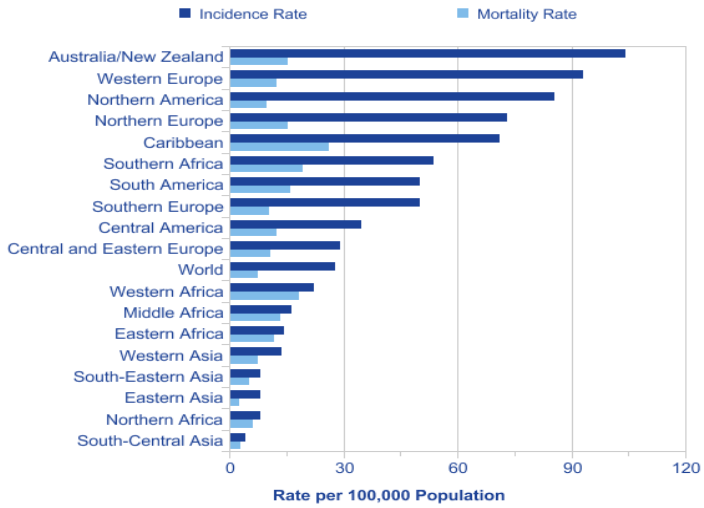



**Figure 4** Age-standardized prostate cancer incidence and mortality rates, World Regions, 2008 Estimates. (International Agency for Research on Cancer, 2010. Available from: http://globocan.iarc.fr/factsheets/cancers/prostate.asp, 12.07.2012)

Compared to the Western countries, the mortality rate is lower in Asia (Figure 4). The difference may be the result of genetic factors and certain protective lifestyle habits, dietary and/or environmental factors. Studies on migrants have shown an increased trend of developing cancer by changed environmental conditions, like e.g. dietary factors, when moving from low incidence to high incidence countries (13).

Although there are several known risk factors for PCa, including age, ethnicity, family history, diet, alcohol, smoking, bodyweight, physical activity, medications, endogenous hormones and diabetes mellitus, no modifiable risk factor has been identified. Men younger than 50 years are exposed to a lower risk of developing PCa, whereas the risk increases during ageing. Some ethnic groups have a higher risk of developing PCa than others. For example,

African and Caribbean men are more likely to develop PCa than Caucasian men. Moreover, a family history of disease may raise the probability of developing PCa. Nutrition high in animal fat (including dairy products) and low in fresh fruit and vegetables is thought to increase the risk of developing PCa. Additionally, a high intake of calcium (such as from dairy foods) may also increase the risk of developing PCa, which may partly explain the lower PCa incidence in Asian countries compared to Western world (11, 12).

### *1.2.2 Pathological staging of PCa*

If the initial prostate specific antigen (PSA) screenings raise suspicion that a patient suffers from PCa, a prostate biopsy may be performed. In the case of a positive biopsy, the stage of cancer progression has to be determined. The TNM (Tumor-Lymph Nodes Metastasis) classification enables both, clinical and histological examination of the prostate. The former is determined using information without surgery and pathological staging based on surgical removal and histological examination of the prostate (14).
This system can be summarized:

| Primary Tumor (T) | |
|---|---|
| TX | Primary tumor cannot be evaluated |
| T0 | No evidence of primary tumor |
| Tis | Carcinoma in situ (CIS; abnormal cells are present but have not spread to neighboring tissue; although not cancer, CIS may become cancer and is sometimes called pre-invasive cancer) |
| T1, T2, T3, T4 | Size and/or extent of the primary tumor |

| Regional Lymph Nodes (N) | |
|---|---|
| NX | Regional lymph nodes cannot be evaluated |
| N0 | No regional lymph node involvement |
| N1, N2, N3 | Involvement of regional lymph nodes (number of lymph nodes and/or extent of spread) |

| Distant Metastasis (M) | |
|---|---|
| MX | Distant metastasis cannot be evaluated |
| M0 | No distant metastasis |
| M1 | Distant metastasis is present |
| M1a | Non-regional lymph node(s) |
| M1b | Bone(s) |
| M1c | Other site(s) with or without bone disease |

### 1.2.3 Grading (Gleason Score)

Prostate biopsy specimens are graded via the Gleason score, a powerful histological tool used to classify prostate carcinomas and to estimate tumor aggressiveness. Thereby, the growth differentiation of the tumor is determined by grading the specimens from the most differentiated to the most undifferentiated architectural pattern (1 to 5) (Figure 5). The most prevalent pattern plus the 2$^{nd}$ most common pattern is summed to yield the Gleason score. Gleason scores 2 to 4 characterize the least aggressive cancers. Intermediate-grade cancers have Gleason scores of 5 and 6 and are with few exceptions slow growing and less aggressive. High Gleason grade cancers have Gleason scores of 7-10. Low and intermediate grade tumors are thought to grow more slowly, whereas a high Gleason score is correlated with a more aggressive tumor. The predominant Gleason score in PCa lesions are 6 and 7. Gleason score 7 can be 3 + 4 or 4 + 3 depending which is the predominant Gleason pattern. Both are considered higher grade PCa, however, the Gleason score 4 + 3 = 7 has a worse prognosis than 3 + 4 = 7 (15).

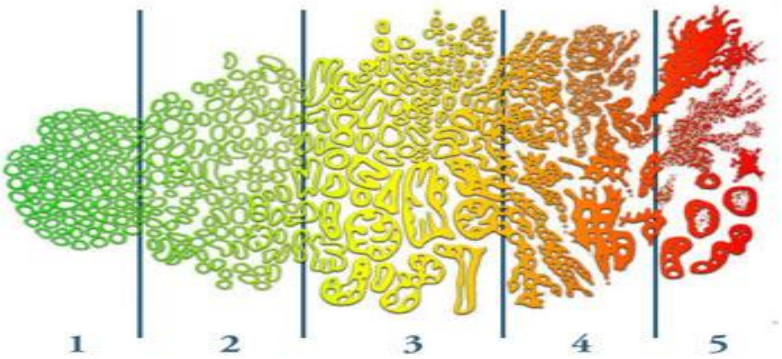



**Figure 5** The histological Gleason grading of prostatic adenocarcinoma. (http://www.stjohnprovidence.org/prostateimplant/Evaluation/, 15.07.2012)

### 1.2.4 Treatment of the PCa

The treatment strategy against PCa depends on the stage of progression. If the tumor has extended beyond the capsule or invaded the seminal vesicle, radiotherapy is the recommended treatment. Besides radiotherapy, radical prostatectomy and cryosurgery are still the most effective options for patients with an organ confined tumor.

#### Radical prostatectomy

The radical prostatectomy (RPE) is the surgical removal of the entire prostate from the urethra and bladder, which are then reconnected. This operation should be considered for

patients with moderately and poorly differentiated localized PCa, at least with a life expectancy of 10 years. There are different techniques available to perform the RPE:

Retropubic RPE: Surgery through an incision in the lower abdomen.
Perineal RPE: Surgery through an incision between the scrotum and the rectum.
Laparascopic RPE: Surgery using a small video camera that is put inside the small incisions.
Robotic-assisted laparoscopic RPE: A computer-assisted surgery, involving several small abdominal incisions used to remove the entire prostate.

The computer-assisted surgery may have some advantages compared to the traditional RPE including smaller incisions, less pain, less bleeding, less risk of infection, faster healing time and shortened hospital stay (16).

### *Radiation therapy*

Radiation therapy, also called X-ray therapy, aims to harm more frequently dividing cancer cells by high levels of locally applied radiation. The damage of healthy cells is reduced due to their lower dividing frequency. Radiation is either applied via a radiotherapy machine or by direct injection of seeds loaded with radioactive isotopes into the cancerous area (brachytherapy). The X-ray therapy is also applied to patients as an additional follow-up therapy after RPE, especially in case of tumor extension beyond the prostate capsule, but also to early stage PCa patients to reduce their symptoms when it is given in combination with androgen deprivation therapy (ADT). Radiation therapy with short-term ADT increases survival in early stage PCa, according a multicenter study (17). The latter was shown to improve a survival benefit in patients by reducing the risk of biochemical PCa relapse (18).

### *Hormone therapy*

Hormone therapy, either androgen ablation or antiandrogen therapy, is based on the reduction of circulating androgens or blockade of the androgen receptor. Androgens are steroid hormones, such as testosterone or androsterone, which control the development and maintenance of masculine characteristics. Androgen receptor is a nuclear receptor activated by binding of the androgens to its ligand-binding domain. Hormone ablation prevents the activation of the androgen receptor, a steroid hormone receptor that regulates genes in diverse cellular processes that play an important role in the function of the prostate.
Despite androgen withdrawal inhibition of androgen receptor activation is also achieved by antagonists such as bicalutamide or enzalutamide. Most studies have shown that androgen ablation by applying androgen receptor antagonist causes a reduction in tumor volume and also an improvement in symptomes in most PCa patients. Hormonal therapy is commonly

used in locally advanced or high-grade PCa. The therapeutic use of various hormones such as estrogen or agents like luteinizing-hormone-releasing hormone (LHRH) analogs, results in blockade of the brain – testis signaling axis, thereby stopping testicles production of androgens. Hormone treatment may also include surgical removal of the testicles (orchiectomy).

### *Chemotherapy*

Chemotherapy is a palliative treatment for metastatic disease. The aim of this therapy is to target fast dividing abnormal cells by cytotoxic drugs. Under androgen ablation therapy most patients develop hormone-refractory PCa, in average after 12-18 months. In the following the most commonly used chemotherapeutic drugs are listed: (www.prostate-cancer.com)

Mitozantrone (Novantrone) is an antineoplastic or anti-tumor antibiotic that is often applied in combination with the steroid prednisone to patients suffering from pain caused by bone metastasis.

Doxorubicin (Adriamycin) is an anti-tumor antibiotic, which damages the nucleus of cells and is derived from the bacterium *Streptomyces peuceticus*.

Vinblastine (Velban) is an antineoplastic vinca alkaloid, which disrupts cell division and is derived from the Madagascar periwinkle.

Paclitaxel (Taxol) is a member of the taxane group, which stabilizes the microtubules and thus blocks cell division. Is a second line taxane for prostate cancer treatment, effective in many tumors that developed docetaxel resistance.

Vinorelbine (Navelbine) is an antineoplastic vinca alkaloid, which disrupts cell division and is derived from the Madagascar periwinkle.

Docetaxel (Taxotere) is another member of the taxane group, which is won from the bark of the Pacific Yew tree. It is currently the state of the art chemotherapy for prostate cancer.

Etoposide's (VP-16) chemotherapeutic concept is based on the inhibition of mitosis in cancerous cells.

Carboplatin (Paraplatin) is an alkylating agent, which kills cancer cells by binding to the DNA and interfering with the cell's repair mechanisms, which leads to cell death.

Estramustine Phosphate (Emcyt) is an alkylating agent, which damages the DNA of the cells and lowers the testosterone production in the body.

### *Watchful waiting (active surveillance)*

This method is recommended to men diagnosed with low grade and slow-growing tumors that have a life expectancy of less than ten years. Active surveillance is based on carefully monitoring by consistent PSA screening (3 months interval), DRE and annual biopsy.

#### *Cryotherapy and HIFU*

Cryotherapy and HIFU (High Intensity Focused Ultrasound) are two further converse therapies applied to PCa patients. While in cryotherapy Argon or liquid Nitrogen are used to freeze the tumor cells, HIFU uses heat to kill cancer cells.

### *1.2.5 Diagnosis*

Measurement of the serum tumor marker prostate-specific antigen (PSA) serves as the current mainstay in PCa detection, whereby high or rising serum levels of PSA are suggestive of prostate disease. Further diagnostic procedures commonly used in PCa detection are Digital Rectal Examination (DRE), Trans-rectal Ultrasound (TRUS), and needle Biopsy. Together those techniques enable detection of early stage cancer, thus increasing the chance of curative treatment of affected patients.

A DRE is performed by rectal palpating the prostate, which enables detecting lumps, enlargements, or callous areas that might indicate a prostate tumor. However, this technique is limited to the examination of the back wall of the prostate gland, so abnormalities located in the middle or front part of the gland cannot be detected. As shown by Selley *et al*. TRUS, a medical imaging technique based on ultrasound, is used in combination with needle biopsy (19). A prostate biopsy may be subsequently performed when TRUS indicates prostate abnormalities or when the PSA measurements raise suspicion of PCa. Other imaging techniques that can be used for PCa diagnosis are computer-tomography (CT) and magnetic resonance imaging (MRI).

### *1.2.6 PSA testing*

In 1987 PSA has been first reported as a marker for PCa (20). In 1991, a study of Washington University introduced PSA screening as a standard method in PCa detection (21). To sum up, PSA testing serves as a tool to identify prostate biopsy, evaluate the response to therapy, determine tumor progression and it has become an important screening method in the detection of PCa (22-24).

#### *Physiologic characteristics of PSA*

The prostate-specific-antigen is a 33kD protein, a member of the human kallikrein family of proteases, and the gene is localized at chromosome 19 (24, 25). PSA as human glandular kallikrein 3 (hKLK3) has an 80% sequence homology with another PCa marker hKLK2, and shows 73% homology with the third kallikrein (hKLK1), which is found primarily in pancreatic and renal tissues. Like many proteases, it plays a crucial role in coagulation processes. PSA

dissolves the seminal fluid coagulum and plays an important role in fertility. The highest amount of PSA is found in the seminal fluid, however, it may also be found in the serum (26-28).

### *Production of PSA*

PSA is found predominantly in prostate epithelial cells and in the seminal fluid (29). Low concentrations of PSA have been identified in urethral glands, endometrium, normal breast tissue, breast milk, salivary gland tissue and male as well as female urine. It can also be detected in serum of women suffering from breast, lung and uterine cancer. Furthermore, serum levels of PSA where shown to be increased in renal cancer patients, however, with a low frequency. (30-32)

The lumen of the prostate contains the highest concentration of PSA in the whole body and reserves a number of barriers to prevent sequestration into the blood circulation. However, infections, inflammations and cancer may bypass these barriers, resulting in a release of PSA to the circulation. In the case of an infection the increased PSA level returns to the reference range after cure.

### *Effects on growth factors*

PSA may also have anti-angiogenic properties inhibiting fibroblast growth factor (FGF-2) and vascular endothelial growth factor-induced endothelial cell proliferation, migration and invasion (33). Another study could demonstrate a concentration-dependent proliferative response of benign prostatic hyperplasia (BPH)-derived stromal cells on insulin-like-growth factor (IGF-1) (34). PSA as a serine protease can cleave insulin-like growth factor-binding protein-3 (IGFBP3), which decreases its affinity for insulin-like growth factor-1 (IGF-1), thus stimulating cellular proliferation like BPH.

### *Factors influencing PSA levels*

The majority of PSA is produced in the transitional zone of the prostate which is associated with benign prostatic hyperplasia (BPH). A relatively small amount of PSA is produced in the peripheral zone, where 80% of PCa tumors originate. For this reason distinguishing between BPH and PCa caused increase of PSA level is difficult (35).

In healthy men serum PSA levels are very low, however, these levels may increase caused by PCa or benign diseases such as prostatitis. Additionally, urinary retention may also lead to altering PSA levels. Whereas the PSA level remains constant after digital rectal examination (DRE), cystoscopy, urethral catheterization and transrectal ultrasonography (TRUS), a vigorous prostate massage can increase PSA by 2-fold.

PSA levels in Africans/African-Americans are higher compared with whites, even after adjusting for age, clinical stage and Gleason score. However, it is not recommended to use this difference as a race-specific range for screening (36).

### *Protein binding*

Serum PSA appears in two different forms. The majority of PSA is attached to blood proteins, whereas a small amount remains unbound, which is the so-called free PSA (fPSA). The ratio between circulating free and total PSA is called percent free PSA. It was shown that this ratio (f/tPSA) is lower in malignant prostatic disorders than in benign ones (37). However, the prostate volume is thought to have an underestimated effect on the f/tPSA (38). Thus, the prostate size is an important variable when selecting a cutoff value for fPSA. Since age is an appreciable factor regarding prostate size, Oesterling *et al* proposed the use of an age-related reference range to detect PCa (39). A large Austrian study showed that f/tPSA ratio determination may benefit the detection of PCa and improve the ability to distinguish between men with PCa and men with BPH (40).

Another test known as complexed PSA (cPSA), directly measures the amount of PSA that is attached to blood proteins (the portion of PSA that is not "free"). It is suggested that cPSA offers the best discriminatory ability when the serum PSA value is higher than 10ng/ml, while the highest specificity of f/tPSA testing is reached at total PSA ranges between 0 and 10ng/ml. To sum up, cPSA compared to tPSA (c/tPSA) offered better specificity at high sensitivities in all tPSA-ranges (41).

### *Age-specific PSA reference ranges*

It is thought that the use of age-related reference ranges would increase both, cancer detection rate in younger men and specificity of PSA testing in older men (42). Reissigl *et al* studied the effect of biopsy rates and PCa detection using age-specific ranges and a PSA cutoff of 4ng/ml (43). They demonstrated that older men have larger tumor volumes compared to younger men with the same PSA level (44). PSA-based screening with low PSA cutoff may increase the detection rate of PCa. However, this would not prevent unnecessary biopsies and treatments. Patients with elevated PSA levels or suspicious DRE findings are specifically recommended to undergo biopsy.
Table 1 demonstrates the age dependency of serum PSA levels used at the Department of Urology at the University Hospital Innsbruck.

| Age range | PSA range |
|---|---|
| below 50 years | 0 – 1.25 ng/ml |
| 50 – 59 years | 0 – 1.75 ng/ml |
| 60 – 69 years | 0 – 2.25 ng/ml |
| over 70 years | 0 – 3.25 ng/ml |

**Table 1** Bisected, Age-Specific PSA Reference Ranges
used at the Department of Urology at the university hospital Innsbruck, Austria. (45)

Age-referenced PSA levels in combination with percent free PSA of 18% were used as biopsy criteria. The following procedures to perform a biopsy are recommended depending on the PSA values. (Figure 6)

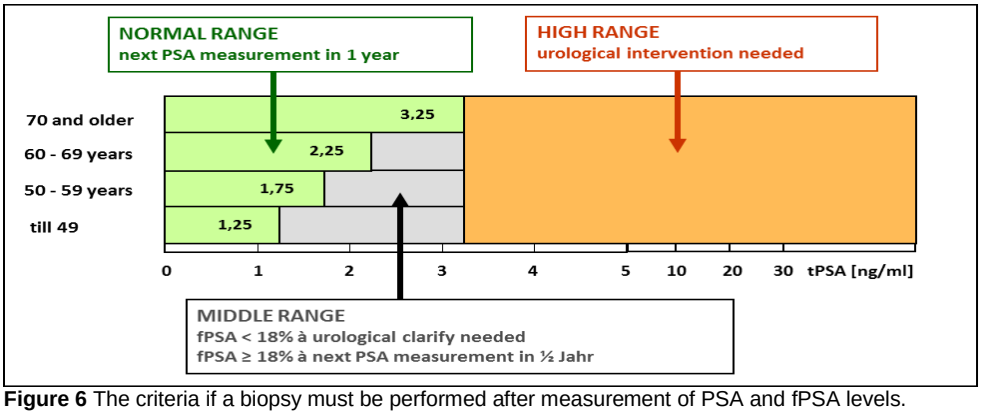



**Figure 6** The criteria if a biopsy must be performed after measurement of PSA and fPSA levels. (http://urolab.uki.at/page.cfm?vpath=klinisches_labor/psa_screening, retrieved 18.05.2012)

### *PSA velocity (PSAV)*

In addition to PSA, other related parameters like PSA-velocity are used to observe changes in PSA levels. The PSA velocity is the alteration of the PSA concentration compared to the PSA level of the preceding year. Values greater than +0.75 ng/ml are found in patients with an increased risk of PCa. A study showed that the PSA velocity may be an important tool to improve the ability of PSA in PCa detection (46) and that it could be associated with Gleason scores and pathologic stage (47). Furthermore, it is suggested that using this concept may improve the cancer detection in men with a low PSA value (48).

## *1.3 Novel Diagnostic biomarkers for PCa*

The National Cancer Institute defines a biomarker as “a biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process or of a condition or disease.” (http://www.cancer.gov/dictionary?cdrid=45618). A biomarker shows further information to currently existing clinical and pathological analysis. The biomarkers predicting PCa can vary from metabolites and chemical products existing in body fluid to genes and proteins in the prostate tissues. In the following some PCa marker genes are described.

### 1.3.1 TMPRSS2-ERG

The gene fusion of transmembrane protease, serine 2 (*TMPRSS2*) and the transcription factor v-ets erythroblastosis virus E26 oncogene homolog (*ERG*) can be detected in about 50% of all PCa patients (49) and account for 90% of PCa fusions (50).

These gene fusions are specific for PCa and can even be detected in precursor lesions, such as prostate intraepithelial neoplasia (PIN) (51). The existence of TMPRSS2-ERG fusion itself is thought to be a potential prognostic PCa biomarker when detected in tissues. Moreover, some studies showed the association of the appearance of the TMPRSS2-ERG fusion with an aggressive progression (49, 52, 53). However, more studies would be needed to approve, whether TMPRSS2-ERG can be used as a trustworthy biomarker in the future of PCa detection.

It has been demonstrated that urine TMPRSS2-ERG in combination with urine PCA3 enhances the usefulness of serum PSA to predict PCa risk (52).

### 1.3.2 PCA3

PCA3 is a prostate tissue-specific, long non-coding RNA that is significantly over-expressed in PCa cases compared with normal or BPH tissues (54, 55). It has been shown that PCA3 may be useful to predict the presence of malignancy in men undergoing repeated prostate biopsy (56, 57).

In comparison to PSA, PCA3 has higher specificity and a better positive and negative predictive value in cancer diagnosis. Moreover, its level is independent of prostate volume (56). However, it is thought that combination of serum PSA and urine PCA3 testing may best improve PCa detection (58).

PCA3 is measured in urine after a prostate massage with digital rectal examination. In 2012, PCA3 screening was accepted by the FDA as a diagnostic test to determine, whether there is a need to repeat a prostate biopsy in men, in which previous biopsies were negative.

(http://www.fda.gov/MedicalDevices/ProductsandMedicalProcedures/DeviceApprovalsandClearances/Recently-ApprovedDevices/ucm294907.html, 18.08.2012)

### 1.3.3 AMACR

An alpha-methylacyl-CoA racemase (AMACR) is an enzyme localized in peroxisomes and mitochondria that is involved in ß-oxidation of dietary branched-chain fatty acids.
Studies have shown that over-expressed AMACR in PCa epithelium may predict the risk of PCa. AMACR plays an important role in fat metabolism and has high tissue specificity for prostate adenocarcinoma (59). Furthermore, there is an association between excessive consumption of branched-chain fatty acids derived from beef and PCa risk (60). Several studies conducted genome-wide screening and demonstrated that the AMACR locus (5p13) is also the location of a PCa susceptibility gene (61, 62). AMACR was identified as a PCa tissue marker (63) and was initially not considered to be suitable for noninvasive detection in urine (64). However, a more recent study could demonstrate the presence of AMACR transcript in urine sediments following prostatic massage (65).

### 1.3.4 MELK

Maternal embryonic leucine zipper kinase (MELK), a serine/threonine-protein kinase involved in various processes, such as cell cycle regulation, apoptosis and splicing regulation, plays a key role in cell proliferation and carcinogenesis. An increase in MELK expression is associated with the malignancy grade of mammary carcinoma, brain and prostate cancer (66-69).
A recent study on an orally-taken MELK-targeting inhibitor pointed out that inhibition of MELK activity may suppress the growth of various types of human cancer. The inhibition was related to the suppression of potential tumor-initiating cells (70).

### 1.3.5 KLK-2

The KLK-2 gene belongs to the kallikrein gene family, which also includes PSA. Similar to PSA, high levels of KLK2 have been found predominantly in prostate as a complementary marker to PSA. Thus, it may play a role for early PCa detection. It was shown to cleave proPSA to generate enzymatically active PSA (71). This suggests that KLK2 plays a physiological role in the regulation of PSA activity.
KLK2 was also detected in urine and other biological fluids. While the distribution of concentration of PSA and KLK2 is similar in seminal plasma and male serum, KLK2 levels in prostate tissue only reach 1-2% of the PSA concentration. Both prostate kallikreins were more frequently expressed in non-cancerous areas than in cancerous prostatic tissue (72).

## 1.4 Exosomes

In general, the term microvesicle includes several types of secreted vesicles. Microvesicles with a size between 100 and 200 nm form multivesicular bodies (MVB) and contain transferrin receptor, a marker involved in endocytosis (73). Recent studies suggests that the size of vesicles is not strictly determined as that of exosomes and they can be between 50-1000nm in diameter (74).

Simply, when MVB fuse with the plasma membrane, these ~50nm buds are released into the extracellular environment. These secreted vesicles are then called exosomes (75). The ubiquitous nature of exosomes in body fluids suggests them to be used as a diagnostic tool in biomarker studies. Urinary exosomes can be examined as possible source of biomarkers.

### 1.4.1 Exosome biogenesis and secretion

Exosomes, first described by Rose Johnstone in the 1970s, are small membrane vesicles. They were shown to contribute to the selective removal of many plasma membrane proteins for example in the reticulocyte, a precursor of the red blood cell (75). Their rounded structure and size (average 50–80 nm), which is similar to previously described exosomes in other studies, was determined via electron microscopy (76-78). Exosomes derived from different tissues may have different molecular and biophysical properties. For example the prostasome (150 to 500 nm) is a semen component that plays a role in male fertility (79, 80).

Exosome biogenesis can be divided into four stages: Initiation, endocytosis, multivesicular bodies (MVBs) formation and secretion. (Figure 7) After accumulation MVBs are either degraded in the lysosome, e.g. by EGF activation, or recycled. They are either recycled at the trans-Golgi network or by being transported to the plasma membrane. The intralumenal endosomal vesicles become exosomes, when the endosomes fuse with the plasma membrane and the vesicles are released. As a consequence, late endosomal compartments are released to the extracellular space as exosomes (81). Another secretion mechanism is the inducible release, which in one report was shown to be triggered by a pathway activated by p53-mediated DNA-damage response. This indicates that tumor suppressor genes may be involved in triggering exosome formation (82). Another study has shown that exosome secretion is a highly regulated, $Ca^{2+}$-dependent process. Monensin (a $Na^{+}/H^{+}$ exchanger) treatment, which increases intracellular $Ca^{2+}$ accumulation, results in increased exosome release (83). Furthermore, low-pH conditions increase the secretion of exosomes and their uptake from recipient cells (84). This may give a possible explanation for the increased secretion of exosomes in cancer patients (85), as the tumor core mass has a low pH value

due to limited vascularization, nutrient and oxygen supply. On the other side, conditions like starvation, stress, rapamycin and hormone treatment induce autophagy which results in inhibition of exosome secretion (86).

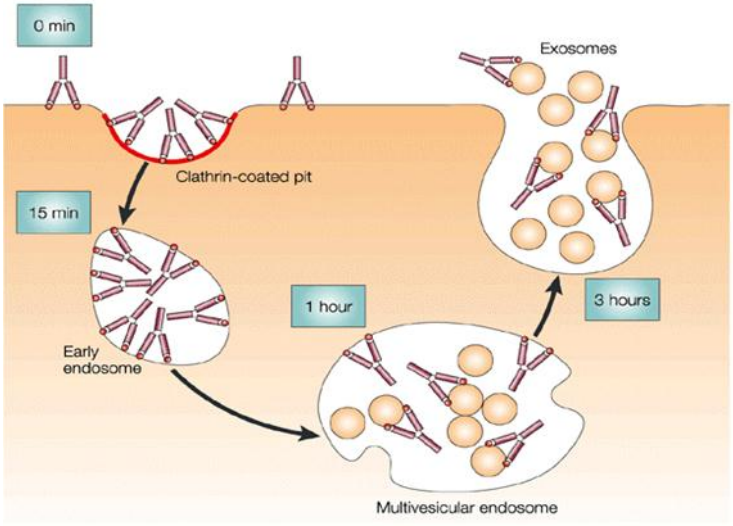



**Figure 7** The secretion of exosomes adapted from Pan *et al* (73)**.**
Using immunoelectron microscopy they inspected the embodying of the transferrin receptor in sheep reticulocytes in-vitro. **0min:** the antibody binds to the cell surface, later is found in clathrin-coated pits, **15min:** the label is found in "early endosomes" after the antibody binds, **1h:** the internalized anti-transferrin-receptor antibody is found in endosomes containing internal vesicles, **3h:** after fusion of multivesicular endosomes with the plasma membrane, transferrin receptors at time 0 are found to be released into the extracellular environment (Source of picture: (87))

Due to the lipid bilayer membrane (88) exosomes are highly stable and thus can be collected not only from cultured cells, but also from many physiological fluids, such as blood, urine, ascites, amniotic fluid (79, 89-92), human cerebrospinal fluid (93) and even from human nasal fluid (94). The released exosomes are transported through the body via the blood circulation (95). Recent biochemical analysis showed that exosomes are also secreted by cells of hematopoietic origin; e.g. B- and T-lymphocytes, platelets, dentritic cells, mast cells, reticulocytes and non-hematopoietic origin; e.g. neurons, intestinal epithelial cells and tumor cells (96-101). After collecting of body fluids or cell culture supernatant exosomes can easily be isolated and pelleted using filtration and differential ultracentrifugation to get rid of unwanted components. However, this procedure does not discriminate exosomes from other small vesicular structures or large protein components. Therefore, exosomes firstly need to be identified by electron microscopy and biochemical analyses to confirm that these vesicles are indeed exosomes. For example, for the separation of cancer derived exosomes, exosome specific antibody coated magnetic beads were used (102). Also, proteomic analysis of exosomes has been recently performed, which involved initial separation of proteins by polyacrylamide gel electrophoresis (PAGE) or high performance liquid chromatography (HPLC), digestion of selected proteins, and finally mass spectrometry to identify the proteins (103).

### *Urinary exosomes*

The first exosomes in human urine were discovered by Pisitkun and colleagues, as an additional component of urine. They analyzed the exosome containing aquaporin-2-integral membrane protein which derived from the urogenital tract (90). The release of prostate exosomes to body fluids such as urine provides an opportunity to access these bioactive vesicles in a non-invasive approach. Phenotypically, individual PCa are often genetically heterogeneous with different tumor foci; moreover, a biopsy taken from one region of the tumor may not be sufficiently informative. Therefore, the transcriptome in secreted PCa exosomes in voided urine has a great importance, giving information about the overall tumor malignancy. Several studies have shown that increased release of exosomes into urine or serum alone is associated with cancer, thus can be implicated as remarkable characteristic of malignancy in PCa patients (82, 104, 105).

Studies on prostate tumor derived microvesicles in cancer patients suggest that exosomes as well as prostasomes may play a role in PCa pathogenesis. It is known that prostasomes are the storage vesicles with their intracellular contents similar to the organelles (106), which release compounds when fusing with the plasma membrane of secretory prostate cells (107). They represent highly ordered and stable structures containing a cholesterol-rich lipid multilayer membrane, in contrast to the exosomes, which usually contain a lipid bilayer membrane (108). However, both vesicles are similar in size, morphology and content, thus it can be summarized that prostasomes are exosomes derived from prostate tissue in a biological setting (109), but so far there are no reports regarding their RNA-content.

### *Exosome contents*

Circulating microvesicles are information carriers of the cell by containing a spectrum of molecules such as signal proteins, peptides, microRNAs, mRNAs and lipids (110). Microarray studies proved the presence of mRNA (of 1300 genes) and miRNAs (more than 100) in exosomes (111). Secreted microvesicle components (e.g. miRNA and mRNA) may affect cellular signaling of target cells by regulation of transcriptional and translational processes. Thus, they became a recent focus in research.

The presence of known and predictable RNAs from various sources has been analyzed mainly by quantitative real time PCR, which allows highly precise signal amplification. An overview of molecules known to be present in exosomes is provided by the “ExoCarta” database. Version 3.1 of ExoCarta contains information on 11.261 proteins, 2.375 mRNAs and 764 miRNAs records obtained from 134 exosomal studies. Table 2 shows a list of 25 proteins that are frequently observed in exosomes.

| | Gene Symbol | Number of times identified | | Gene Symbol | Number of times identified | | Gene Symbol | Number of times identified | | Gene Symbol | Number of times identified | | Gene Symbol | Number of times identified |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | HSPA8 | 52 | 6 | CD81 | 39 | 11 | PKM2 | 33 | 16 | YWHAZ | 31 | 21 | ALDOA | 30 |
| 2 | CD9 | 50 | 7 | ANXA2 | 37 | 12 | YWHAE | 32 | 17 | EEF2 | 31 | 22 | MSN | 29 |
| 3 | GAPDH | 48 | 8 | ENO1 | 36 | 13 | SDCBP | 32 | 18 | ACTG1 | 31 | 23 | ANXA5 | 29 |
| 4 | ACTB | 43 | 9 | HSP90AA1 | 34 | 14 | PDCD6IP | 32 | 19 | LDHA | 30 | 24 | PGK1 | 28 |
| 5 | CD63 | 41 | 10 | EEF1A1 | 34 | 15 | ALB | 32 | 20 | HSP90AB1 | 30 | 25 | CFL1 | 28 |

**Table 2** Often identified exosomal proteins.
(http://exocarta.org/exosome_markers, 25.08.2012)

**The lipid composition** of the secreted exosomes is similar to the plasma membrane of the originating cells (112), consisting of phosphatidic acid, phosphatidylserine, cholesterol, ceramide, sphingomyelin and lipid raft associated proteins. Thus, the lipid bilayer protects the exosomal genetic information from degradation.

**The protein composition** of exosomes is diverse. Since the exosomes are formed during budding of the plasma membrane, these proteins are generally cell surface receptors and components of the endocytic pathway. Protein analysis can be performed via different methods, like mass spectrometry, western blotting, immune electron microscopy and flow cytometry (112). It has been recently shown that exosomes contain selected cytosolic proteins, such as differentially expressed heat shock proteins (Hsp). Hsp60 was shown to be only expressed in tumor cell line-derived exosomes (113). However, Hsp70 was detected in both, exosomes of tumor and non-tumor cell lines.

### *mRNA in exosomes*

Exosomes are also known to contain mRNA, although less compared to the small RNA components (114). Later, Hong *et al.* have also identified over 11.000 distinct mRNAs in the exosomes derived from colorectal cancer cell line, 2% of which are found to be overexpressed (115). The overexpressed RNAs are involved in cellular processes such as cell division or cell cycle. In 2007, Valadi *et al.* were the first to discover intercellular RNA transport through exosomes by protein expression analysis of mouse mast cells, demonstrating that the RNA from mast cell exosomes is transferable to other mouse and human mast cells. It was shown that after transfer of mouse exosomal RNA to human mast cells, new mouse proteins were found in the recipient cells, supporting that transferred exosomal mRNA can be translated after entering another cell. This means that the exosomal RNAs from donor cells taken up by recipient cells are functional and can be actively translated into protein (111).

Many studies have demonstrated that exosomes have a signal function in addition to intercellular transfer of RNA (111, 116, 117). The cells communicate with the neighboring cells by delivering RNA-signals. This RNA is most likely transferred from one cell to another, by fusion of the exosome and the recipient cell's membrane. The uptake process is enabled by selective recognition of the RNA carrying exosomes by surface molecules of the recipient cells. Moreover, a recent affymetrix microarray analysis found that exosomes secreted under oxidative stress may induce tolerance against further stress in recipient cells (118). Furthermore, it was shown that stress conditions like UV-light affect exosomal mRNA content.

Exosomal mRNAs can be dysregulated in disease status. While mRNA expression patterns are partially defined in most cell types under normal conditions, aberrant expression of certain mRNAs has been described in diverse human diseases. 4700 distinct mRNAs could be detected in exosomes of glioblastoma cells (119), whereby approximately 8% of the exosomal mRNAs were shown to be upregulated more than five-fold compared to the corresponding non-malignant cells. Many of these mRNAs are known to play a role in angiogenesis, cell proliferation, immune response, migration and histone modification.

### *miRNA in exosomes*

miRNAs, approximately 23 nucleotides in length, are noncoding nucleic acids that regulate the gene expression by binding to their target molecules like mRNAs. This interaction may result in decreased stability of mRNAs and/or in decreased translation of target mRNAs (120). Using microarray technology, Valadi *et al.* have shown that exosomes contain mRNAs as well as miRNAs. The miRNAs circulating in exosomes can also be transferred to recipient cells and there regulate gene expression by changing mRNA throughput (121, 122).

Due to their simplicity, specificity and stability in various body fluids miRNAs have a high clinical diagnostic potential as biomarkers. Furthermore, many miRNAs which play an important role in cancer, such as let-7, miR-1, miR-15, miR-16 and miR-375 have been found in exosomes. It has been also confirmed by other researchers that certain cancer types are associated with specific miRNAs, e.g. ovarian and glioblastoma (miR-21), lung cancer (let-7), thus presenting tumor specific miR biomarkers (102, 119, 123).

## 1.5 Aim of the study

Using PSA measurements is the current mainstay in PCa detection. However, due to the non-specific nature of serum PSA new markers are needed. Urine provides an easily usable and available sample source to investigate prostate associated markers due to its direct association to urinary tract organs. Furthermore, urine derived prostate exosomes may show an adequate reserve of the transcriptome from prostate tumors. Due to their stable structure, exosomes protect RNA from degradation in avoided urine, and in this way RNA integrity remains conserved. Since the mRNA signature of tumor cell derived exosomes represents the current cell activity at a particular time, measurement of exosomal RNA markers would allow to trace tumor activity and characteristics. Thus, the determination of mRNA-contained PCa derived exosomes could be used for diagnosis, but also for prognosis. In this thesis, a pilot study is presented with the aim of evaluating detection and analysis of possible PCa markers in urinary exosomes. The establishment of markers from urinary exosomes could be a useful tool to diagnose PCa earlier and more specifically, and also to monitor response to treatment. Based on this idea, we evaluated a candidate urine marker panel measuring the expression level of more than 30 mRNA transcripts using real time RT-PCR (qPCR). Exosomes isolated from frozen archive urine samples were isolated by ultrafiltration and RNAs were isolated, preamplified and subjected to PCR analysis. Some of the prostate derived genes chosen in this study were already described in the literature as possible PCa markers, e.g. *PCA3*, *AMACR*; other candidate markers derived from previous studies done by our group. In cooperation with the Division of Genetic Epidemiology at the Medical University of Innsbruck a high-through-put RT-PCR project using 384 well plates was designed. By using a pipetting robot, 26880 single PCR reactions were performed with samples derived from a total of 60 prostate specimens. Single gene expression profiles were analyzed by SPSS using the Mann-Whitney-U Test. Furthermore, a machine learning system was used to investigate the performance of marker combinations.

Simply, in this study we aimed to:

i. Establish a reproducible protocol for urine exosome RNA analysis,
ii. Analyze the expression of a panel of potential PCa mRNA markers in urine samples,
iii. Evaluate the potential of urine exosome RNA markers as a tool to help in the prediction of a prostate tumor and thus improve PCa diagnosis and reduce false-negative prostate biopsies.

# 2 MATERIALs and METHODs

## 2.1 Preparation of the urine specimens for exosome isolation

Archival urine samples, which were collected after routine digital rectal examination (DRE) and stored frozen for 5-7 years were obtained from 30 benign patients (BE, criteria: elevated serum PSA, tumor-negative prostate biopsy), 20 low grade PCa patients (LG, criteria: positive biopsy, Gleason score ≤3+4 in radical prostatectomy specimen) and 10 high grade PCa patients (HG, criteria: positive biopsy, Gleason score ≥4+3 in radical prostatectomy specimen).

In the 30 benign patients the average serum PSA concentration was 4.4g/L (median 2.85, range 0.43–31.69) in the 30 cancer patients 4.3g/L (median 3.42, range 1.15-21.33). For the subject group studied, 52 of 60 urine specimens yielded sufficient RNA for statistical analysis, corresponding to an informative specimen rate of 87%. The protocols for urine and biopsy sample collection were established at the Clinic of Urology, Innsbruck.

### 2.1.1 Exosome isolation

Ten milliliter of frozen urine samples were thawed, vortexed and centrifuged at 500 g at 10$^{o}$C for 10 min. The supernatants were transferred to fresh tubes containing 1 mM TCEP (*tris 2-carboxyethyl phosphine*) from *SIGMA*, which reduces the protein disulfide bonds. The samples were centrifuged at 16.000g for 20mins at 10$^{o}$C. The supernatants were then filtered through 0.45µm filter, followed by transferring to the Vivaspin 20 (300.000 MW) filtration column (for separation of exosomes from supernatant of cell cultures, the Vivaspin 1.000.000 MW filtration column was used). For exosome enrichment the samples were centrifuged in the column for 15-20mins at 1500g, 10°C to a volume of about 200 µl. If the concentrated volume exceeds 250 µl the centrifuge step was repeated.

## 2.2 RNA isolation

The isolation of sufficient and high quality ribonucleic acid (RNA) from old urine was a challenge. Several different methods were tested to improve RNA yield, enhance purity and facilitate measurement of low abundance mRNAs. To optimize the protocol, several different kits and methods were tested. For the extraction of total RNA:

I. RNA isolation using the QIAamp Circulating Nucleic Acid Kit from *QIAGEN* (Vacuum method)
II. Extraction with Trizol/chloroform followed by isopropanol precipitation (Trizol method) from *SIGMA*
III. Extraction using the *Zymo Research* Trizol Kit (Spin-column method)

RNA amplification methods:

I. Transplex Complete Whole Transcriptome Amplification Kit (WTA) from *SIGMA*,
II. QuantiTect Whole Transcriptome Kit from *QIAGEN*,
III. TaqMan Preamplification Kit from *Applied Biosystems*

Purification methods used after amplification:

I. DNA Trizol Isolation from *SIGMA*
II. QIAquick PCR Purification Kit from *QIAGEN*

### *2.2.1 QIAamp Circulating Nucleic Acid Kit (Vacuum method)*

QIAamp Circulating Nucleic Acid Kit from *QIAGEN* is based on DNA binding to a silica-based membrane. Tube extenders and vacuum processing on the spin columns enable starting sample volumes of up to 5 ml (Figure 8).

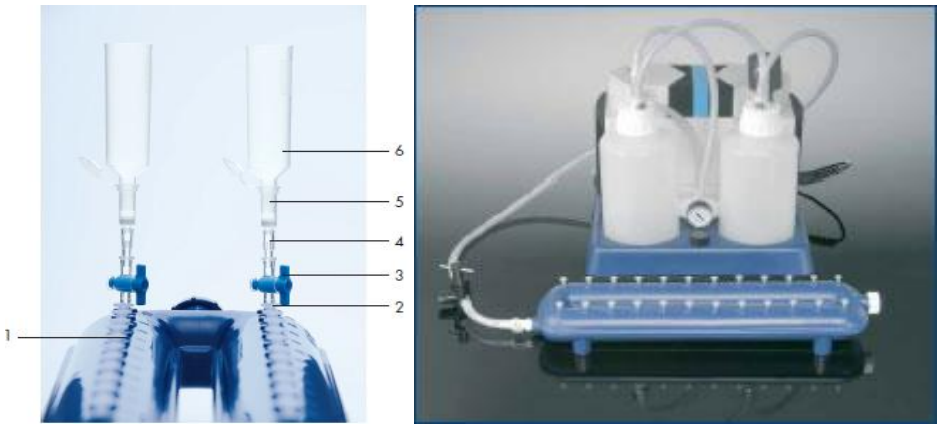



**Figure 8** Setting up the QIAvac 24 Plus with QIAamp Mini columns using VacValves, VacConnectors, and Tube Extenders. (source: QIAamp Circulating Nucleic Acid Handbook, *QIAGEN)*

1. QIAvac 24 Plus vacuum manifold
2. Luer slot of the QIAvac 24 Plus (closed with luer plug)
3. VacValve (Must be purchased separately)
4. VacConnector
5. QIAamp Mini column
6. Tube Extender

The flow-through part of the urine (about 5ml) from the Vivaspin concentrator was transferred onto the tube extender, and the circulating nucleic acids were adsorbed from the large

volume onto the small silica membrane as the urine is drawn through by vacuum pressure. Although the flow-through part of the urine should be exosome-free after the Vivaspin concentrators, samples were tested with this method to determine, whether there was still exosome-free RNA.

### 2.2.2 TRI Reagent RNA Isolation

#### Background

TRI Reagent combines phenol and guanidine thiocyanate in a mono-phase solution to facilitate the immediate and most effective inhibition of RNase activity. The sample is homogenized in Trizol and the homogenate is separated into aqueous and organic phases by addition of chloroform and centrifugation. RNA remains exclusively in the aqueous phase, DNA in the interphase and proteins in the organic phase. RNA is precipitated from the aqueous phase by addition of isopropanol, washed with ethanol and solubilized. DNA and proteins are sequentially precipitated from the interphase and the organic phase with ethanol and isopropanol, washed with ethanol and solubilized.

#### TRIzol Protocol

1ml TRI Reagent from SIGMA was added to the exosome-concentrated Vivaspin tubes, vortexed and incubated for 5min at room temperature to complete dissociation of nucleoprotein complexes. 200µl chloroform was added per 1ml of TRI Reagent. The mixture was shaken for 1min, incubated at room temperature for 2min and centrifuged at 14.000rpm in an Eppendorf centrifuge for 10min at 4°C. After centrifugation, the mixture separates into a lower red phenol-chloroform organic phase, an interphase and the colorless upper aqueous phase. RNA remains exclusively in the aqueous phase, whereas DNA and proteins are in the interphase and organic phase, respectively. Following the transfer of the aqueous phase to a fresh tube, isopropanol was added to the supernatant (amount of supernatant x 0.9 = amount of isopropanol). As the precipitated RNA was expected to be a small amount, 20µg of glycogen was added as a carrier, and subsequently the sample was incubated at -20°C o/n. The samples were pelleted by centrifugation for 10min at 17.000g at 4°C and the pellets washed two times in 1ml 70% EtOH and centrifuged again under the same conditions thereafter. Finally, the pellet was air-dried, dissolved ŧŧ in 20µl of nuclease free water and kept at -80°C until use.

### 2.2.3 Zymo Research Trizol RNA Purification Kit

This method combines the selective binding properties of a silica-based membrane with the speed of microspin technology.

#### Protocol

RNA isolation from supernatant was performed using the kit *DIRECT-Zol RNA Mini Prep* from *Zymo Research* according to the manufacturer's instructions, which is very simple: Samples in TRI-Reagent were transferred to the spin columns and subsequently spun, washed and eluted from the column in 30µl (2x15µl) of RNase-free water. The RNA concentration was measured using spectrophotometer and the samples were stored at -80°C or immediately transcribed to cDNA.

## 2.3 Amplification of the transcript

Due to the generally very low RNA yield isolated from urine exosome (estimated to be lower than 20ng per sample), several amplification methods were tested for unbiased amplification of RNA. These methods were:

### 2.3.1 Transplex Complete Whole Transcriptome Amplification Kit (WTA)

TransPlex WTA creates a cDNA library from the isolated RNA template and then amplifies the library in a single PCR reaction. To synthesize the cDNA library, sample RNA was incubated with reverse transcriptase and non-self-complementary primers consisted of random 3' and 5' ends. Annealed primers are extended by WTA polymerase, thus new templates for continued primer annealing and extension are created from the RNA. Finally, universal-primer PCR amplifies the cDNA library to produce amplified products. Afterwards DNA had to be purified. Two purification methods were tested after RNA amplification. One of them was the DNA isolation with TRI-reagent. With this purification method DNA was isolated from the interphase and phenol phase separated from the initial homogenate. Following precipitation and washing steps, DNA had to be resuspended in NaOH, and pH adjusted. Because of the difficulty to re-dissolve DNA in water after the trizol reaction, a weak basic solvent like 8mM NaOH was applied. For storage and for some downstream applications the basic pH can be a problem, therefore 1M HEPES was added to adjust a neutral pH.

Another purification method tested was the QIAquick PCR Purification Kit. Using the silica-membrane-based purification method PCR products can bind to the membrane; and to clean

them from PCR primers, nucleotides, buffer components and enzymes a wash buffer is used, then the purified PCR products are eluted from the fiber matrix.

### 2.3.2 QuantiTect Whole Transcriptome Kit

The QuantiTect Whole Transcriptome Kit contains reverse transcription, ligation and whole transcriptome amplification steps. Cleaning up of cDNA is not necessary as the amplified DNA is ready for RT-PCR. 5µl of total RNA was used to amplify the transcript.
The third amplification method, the TaqMan Pre-amplification Kit from *Applied Biosystems*, will be described in more detail below, because it was the amplification method chosen for this study.

## 2.4 Reverse transcription (RT) and pre-amplification

The iScript cDNA Synthesis Kit (Bio-Rad) was used for reverse transcription of mRNAs. The 15µl RT reaction including 10.5µl of total RNA, 3µl *5X iScript* select reaction mix*,* 1µl random primers and 0.5µl of *iScript* reverse transcriptase was incubated at 25°C for 5min, then at 42°C for 30min and subsequently at 85°C for 5min to heat-inactivate the reverse transcriptase.

### 2.4.1 TaqMan Pre-amplification Kit from Applied Biosystems

The amount of cDNA obtained from exosomes in urine was not sufficient to perform the whole study and had to be amplified first. Amplification is based on a PCR reaction using a predesigned primer pool consisting of the primers of all genes of interest in this study. The preamplification was performed according to the TaqMan® PreAmp Master Mix Kit protocol (Applied Biosystems, Cat.4391128). The primer pool was prepared by combining the primers of 40 real-time PCR primer kits (25 from TaqMan Gene Expression Assays (*ABI* assay 20X) and 15 lab-designed primer-pairs) into a single tube. The pooled assays were diluted with water to get a 0.2X final concentration. (Table 3) The 30µl preamplification reactions contained 15µl of 2× TaqMan PreAmp Master Mix, 7.5µl of 0.2× primer pool mix and 7.5µl of cDNA sample. The reactions were incubated in the *ABI* thermocycler for 10min at 95°C followed by 17 cycles of 95°C for 15 seconds and 60°C for 4 min and finally an extension at 60°C for 7 min.

| TaqMan | Concentration Stock (µM) | End concentration in RT-PCR (µM) | End concentration in PreAmp (0,2X) µM |
|---|---|---|---|
| Primer F | 100 | 0,8 | 0,04 |
| Primer R | 100 | 0,8 | 0,04 |
| Probe | 100 | 0,15 | 0 |
| ABI 20x | 20X | 1 | 0,05X |

**Table 3** TaqMan gene expression assay for PreAmplification.
Primer F = forward primer; primer R = reverse primer.

## 2.5 Real time PCR

Real-time PCR (RT-PCR) reactions were carried out on the ABI7900 instrument from Applied Biosystems in quadruplicates for each sample. A 10µl PCR reaction contained 5µl of 2x TaqMan Master mix, 0.5µl of TaqMan gene expression Assay Mix (primers and probe), 3µl of 1:50 diluted cDNA and 1.5µl of RNAse-free H2O. For self-designed assays 0.16µl of each primer (50µM) and 0.015µl of probe (100µM) were used. The probes were labeled at the 5'-end with 6-carboxy-fluorescein (FAM) and at the 3'-end with 6-carboxy-tetramethylrodamine (TAMRA). FAM functions as the reporter dye and TAMRA as the quencher dye.
The PCR reaction conditions were as follows: 50°C for 2min, 95°C for 10min followed by 40 cycles of 95°C for 15s and 60°C for 1min. The PCR-efficiency for each gene was calculated according to the equation E = -1+10(-1/slope) using the Efficiency Calculator from Agilent Technologies. (http://www.genomics.agilent.com/CalculatorPopupWindow.aspx?CalID=8 )

The house-keeping gene RHOA was chosen to normalize specific mRNA expression levels against total RNA. The reference control sample was the exosome RNA isolated in the lab from mixed cell culture supernatants of 6 cell lines (DuCap, LnCap, PC3, Du145, 22RV1 and LnCap). The relative expression ratio of a gene of interest was computed on the basis of its RT-PCR efficiency and the Ct-value difference (to the housekeeping gene) according to the "ratio" formula. To calculate real time PCR results the simple method developed by M.W. Pfaffl was used (124).

$$\text{ratio target gene expression (experimental/control)} = \frac{\text{fold change in target gene expression (expt/control)}}{\text{fold change in reference gene expression (expt/control)}}$$

### 2.5.1 Sample Sheet for the high-throughput RT-PCR

All RT-PCR runs were performed in quadruplicate via automatic pipetting. Subsequently the reactions were transferred from 96-well to a 384-well plate. Every time the same sample sheet format was used (Table 4).

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | PTC-P | Be 4 | Be 10 | Be 16 | Be 22 | Be 28 | Ca 4 | Ca 10 | Ca 16 | Ca 22 | Ca 28 | P4 |
| B | NTC | Be 5 | Be 11 | Be 17 | Be 23 | Be 29 | Ca 5 | Ca 11 | Ca 17 | Ca 23 | Ca 29 | P5 |
| C | PTC-S | Be 6 | Be 12 | Be 18 | Be 24 | Be 30 | Ca 6 | Ca 12 | Ca 18 | Ca 24 | Ca 30 | P6 |
| D | Be 1 | Be 7 | Be 13 | Be 19 | Be 25 | Ca 1 | Ca 7 | Ca 13 | Ca 19 | Ca 25 | P1 | PTC-P (1:10) |
| E | Be 2 | Be 8 | Be 14 | Be 20 | Be 26 | Ca 2 | Ca 8 | Ca 14 | Ca 20 | Ca 26 | P2 | PTC-P (1:100) |
| F | Be 3 | Be 9 | Be 15 | Be 21 | Be 27 | Ca 3 | Ca 9 | Ca 15 | Ca 21 | Ca 27 | P3 | PTC-P (1:1000) |
| G | | | | | | | | | | | | |
| H | | | | | | | | | | | | |

**Table 4** Sample sheet created for mRNA detection, including positive and negative controls.

P1: DuCap cells, P2: LnCap cells, P3: EP156T cells

P4: PC-3 cells, P5: Du145 cells, P6: RWPE1 cells

PTC-S: Positive control, exosome RNA from mixed cell culture supernatants of 6 cell lines

(DuCap, LnCap, PC3, Du145, 22RV1, LnCaP)

NTC: Negative control, only water

PTC-P: Positive control, RNA from mixed cell pellets of 12 cell lines

(LnCap, DuCap, LAPC4, 22RV1, PC3, Du145, MDA-Pca-2b, cRL, BPH, EP-156T, fibroblast PM-151, JEG3)

PTC-P (1:10, 1:100, 1:1000): Dilution of 1:10, 1:100, 1:1000 times of mixed cell pellet to check the PCR efficiency.

### 2.5.2 mRNA genes

The 30 genes and transcript variants including well-known prostate cancer genes (e.g. PCA3, TMPRSS2-ERG, AMACR) were chosen according to previous studies of our group and from the literature (Table 5).

| | | GENE | Primer Sequence/ Assay ID by *ABI* | | | GENE | Primer Sequence/ Assay ID by *ABI* |
|---|---|---|---|---|---|---|---|
| 1 | Prostate specific | KLK2 | Hs00428383_m1 | 17 | From our studies | GPR116 | Hs00391810_m1 |
| 2 | Prostate specific | PCA3 | Hs01371939_g1 | 18 | From our studies | STEAP4 | Hs00226415_m1 |
| 3 | Prostate specific | PSA | P: 5'- CACAGCTGCCCACTGCATCAGGA-3'BHQ1<br>F: 5'-GTC TGC GGC GGT GTT CTG-3'<br>R: 5'-TGC CGA CCC AGC AAG ATC-3' | 19 | From our studies | BAG1-D[4)] | P: 5'TGATGATGGCAGAAATCTCACCATTTTCGATG-3'<br>F: 5'-TTGGACACTCTCAAACACCTGGACA-3'<br>R: 5'-CGAGGGAAGACTGCCCATGAGC-3' |
| 4 | House keeping gene | HPRT1 | P: 5'-TCAAGGTCGCAAGCTTGCTGGTGAAAAGGA-3'<br>F: 5'-GCTTTCCTTGGTCAGGCAGTA-3'<br>R: 5'-GTCTGGCTTATATCCAACACTTCGT-3' | 20 | From our studies | BAG1-wt[4)] | P: 5'-TGGCCCAGGTTGTTGAAGAGGTCA-3'<br>F: 5'-AGGGCAGCAGTGAACCAGTT-3'<br>R: 5'-TCCCAATTAACATGACCCGG-3' |
| 5 | House keeping gene | TBP | P: 5'-TCTTCACTCTTGGCTCCTGTGCACA-3'<br>F: 5-'CACGAACCACGGCACTGATT-3'<br>R: 5-'TTTTCTTGCTGCCAGTCTGGAC-3' | 21 | From our studies | AGR2-Sh[5)] | P: 5'-TTGCTCCTTGTGGCCCTCTCC-3'<br>F: 5'-CGACTCACACAAGGCAGGT-3'<br>R: 5'-GCTCCAGGTTTGACTGTGG-3' |
| 6 | House keeping gene | RHOA | Hs00357608_m1 | 22 | From our studies | AGR2-Lt[5)] | P: 5'-TGCTGAGTCAAGCTTCTCAAAGCAAG-3'<br>F: 5'-GCCAACAGACAACCCAAAGT-3'<br>R: 5'-GCAAGAATGCTGACACTGGA-3' |
| 7 | House keeping gene | PA2G4 | Hs00854538_g1 | 23 | From our studies | PAGE4 | Hs00199655_m1 |
| 8 | House keeping gene | ACTB | Hs99999903_m1 | 24 | From our studies | ERG[3)] | Hs01554634_m1 |
| 9 | From literature | MME | Hs00153510_m1 | 25 | From our studies | BLVRB | Hs00355972_m1 |
| 10 | From literature | CLU | Hs00156548_m1 | 26 | From our studies | GNMT | Hs00219089_m1 |
| 11 | From literature | GOLM1 | Hs00213061_m1 | 27 | From our studies | ANXA2 | Hs01561520_m1 |
| 12 | From literature | F3 | Hs00175225_m1 | 28 | From our studies | TFF3 | Hs00902278_m1 |
| 13 | From literature | TMSB15A | Hs00751699_s1 | 29 | From our studies | MELK | Hs00207681_m1 |
| 14 | From literature | FOLH1 | Hs00379515_m1 | 30 | From our studies | NPM1 | Hs02339479_g1 |
| 15 | From literature | TMPRSS2[1)] | Hs01120965_m1 | 31 | From our studies | VDAC1 | Hs01631624_gH |
| 16 | From literature | AMACR | Hs02786742_s1 | 32 | From our studies | PDIA3 | Hs00607126_m1 |
| 33 | From literature | TMPRSS2-ERG – Fusion[2)] | P:5'-AGCGCGGCAGGAAGCCTTATCAGTT-3'<br>F:5'-TAGGCGCGAGCTAAGCAGGAG-3'<br>R: 5'-GTAGGCACACTCAAACAACGACTGG-3' | | | | |

**Table 5** mRNA genes chosen for the study.
For self-designed real-time PSA taqman assays primers and probe sequences are listed, for purchased assay kits the assay identifiers (Life Technologies) are given. **1)** The target sequence of the TMPRSS2 assay is located in the 3'region of the mRNA, the assay determines only the normal gene transcript **2)** Primers and probe for TMPRSS2 fusion gene detects the most frequent fusion variant (125). **3)** The target sequence of the ERG assay is located in the 3'region of the mRNA, the assay determines the normal wild-type ERG and the fusion gene transcript. **4)** BAG1-D: Splice variant D of BAG1 gene, BAG1-wt: wild type of BAG1 gene. **5)** AGR2-Sh: Short transcript of AGR2 gene, AGR2-Lt: Long transcript of AGR2 gene.

### 2.5.3 Filtering of inconsistent data

#### Consistency of CV-value

The coefficient of variation (CV) is defined as the ratio of the standard deviation (STDEV) to the mean. Since each sample was replicated four times in real time PCR, the consistency of values was checked by the CV-value. Single values bigger than 5% difference from mean were discarded.

$$CV = \frac{STDEV}{MEAN\ VALUE}$$

#### Three undetermined values

For those samples with only one signal value and 3 “undetermined” values in the quadruplicated PCR, the undetermined values were considered as 40 Ct. Those one-signal samples were averaged together with three 40 Ct-values.
[(3 x 40 Ct) + (one signal Ct)] / 4

#### Four undetermined values

In order to avoid elimination of samples with “undetermined” values from statistical analysis a high Ct value was assigned to these samples. For samples with 4 “undetermined” values in the, 15 Ct was added to the Ct-value of RHOA, the house-keeping gene of that sample to generate the assigned Ct value. Afterwards those values were checked, whether they were indeed outside the range of “real” measured values, which should not be lower than 40.
(Ct of RHOA + 15)

## 2.6 Statistical analysis

### 2.6.1 Outliers

An outlier is an observation that is numerically distant from the rest of the data. It had to be discarded because of indicating of the analysis error. The significant outliers were identified by *Grubbs' test Quick calculator*, is a statistical test used to detect outliers in a univariate data set assumed to come from a normally distributed population. Subsequently outliers were excluded from statistical analysis. (http://www.graphpad.com/quickcalcs/Grubbs1.cfm)

(source: http://www.jmp.com/support/downloads/jmp_scripting_library/utility/grubbs_outlier.shtml, 07.10.2013)

### 2.6.2 Comparison of the groups

The IBM SPSS Statistics20 was used to analyze the data. Results were expressed as means ± standard deviation. P values of <0.05 were considered statistically significant.

The data was first assessed by the Kruskal-Wallis test. The Kruskal-Wallis test is the nonparametric method allowing the comparison of more than two independent groups. The parametric equivalent of the Kruskal-Wallis test is the one-way analysis of variance (ANOVA). To compare three or more sets of values from different groups, the groups had to be categorized. In this study 3 groups/categories were named Be (benign), LGSc (low Gleason score) and HGSc (high Gleason score). To compare the benign group to both cancer groups, we defined another category as Be vs Ca (cancer).

The Kruskal-Wallis test evaluates differences in medians among 3 groups (Be vs LGSc vs HGSc and Be vs Ca) but it does not identify where the difference is or how much significance there is. If this test was significant, the Mann-Whitney U test was used to compare pairs of group medians (Be vs LGSc, Be vs HGSc, LGSc vs HGSc and Be vs Ca). The Bonferroni method was used to correct for multiple testing.

# 3 RESULTS

RNA is an important target molecule for the analysis of gene expression and regulation. It plays numerous roles in normal cellular processes and disease states. RNA isolation from cells permits analysis of the current cell activity at a particular time, because only the genes which are currently expressed in the cell are present as RNA at the moment of isolation. In this study, to test the potential of exosomes as a source of PCa mRNA markers, we established a protocol for urine exosome RNA analysis using a multiplex PCR technique. Since RNA was extracted from archive ("old") frozen urine specimens, choosing the appropriate method for optimized RNA purification was the key factor for this study.

## 3.1 Optimization of RNA isolation

In order to develop an optimized protocol, three different total RNA extraction methods and three different RNA amplification protocols were compared. (see Materials and Methods)
The evaluation of the feasibility of the methods was based on real-time RT-PCR detection of the expression of highly expressed prostate specific genes PSA and NPM1.

The QIAamp Circulating Nucleic Acid Kit, the classical TRI-reagent method and the Zymo Research Trizol RNA Purification Kit were tested for optimized RNA-extraction. As shown in Figure 9, 4 urine samples were chosen for RNA isolation. No expression of both PSA and NPM1 genes was found in urine samples 2 and 4, probably due to low RNA content and/or RNA degradation. Whereas the QIAamp Circulating Nucleic Acid Kit (vacuum) showed no or low expression of the genes in most samples, the manual TRI-reagent method (trizol) and the Zymo Research Trizol RNA Purification kit (spin column) worked equally well. Due to the fact that the manual TRI-reagent method is more labor-intense, especially for a large number samples, the Zymo Research Trizol RNA Purification Kit was finally chosen for RNA isolation in this study.

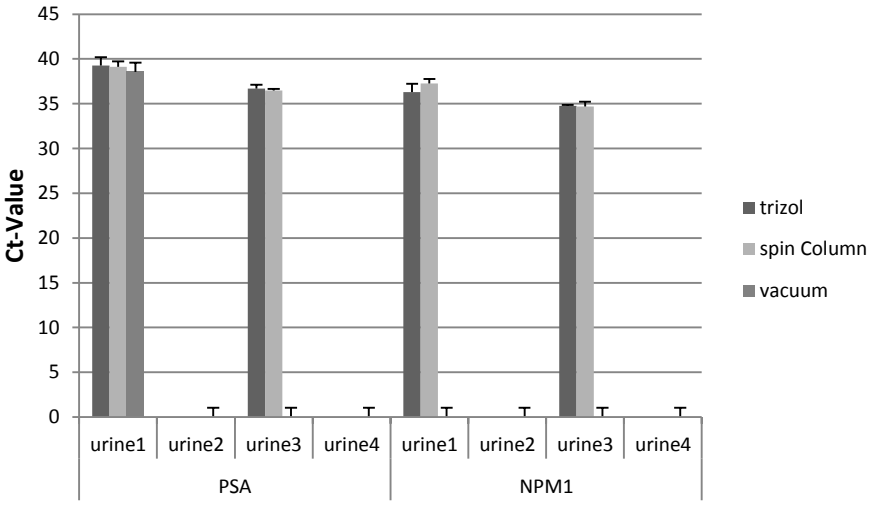



**Figure 9** The comparison of different RNA isolation methods.
Total RNA from 4 urine samples were isolated with three different methods. PSA and NPM1 were chosen for the analysis of RNA-expression. Trizol: manual RNA extraction using TRI-reagent, Spin column: Zymo Research Trizol RNA Purification Kit, Vacuum: QIAamp Circulating Nucleic Acid Kit. According to the graph, the 2. and 4. urine samples had no RNA.

The amount of free-circulating nucleic acids isolated from biological samples like urine are normally very low and are not sufficient for a multiplex analysis. Therefore, several methods were tested for unbiased amplification of RNA.

The whole transcriptome amplification (WTA) kit (*SIGMA)* and the QuantiTect Whole Transcriptome Kit (*QIAGEN)* were used for amplification of RNA from urine and cell-line supernatant controls (Figure 10). The products as well as unamplified cDNA samples were analyzed and compared by RT-PCR amplification of PSA and NPM1. As shown in Figure 10, both the Whole transcriptome amplification kit and the QuantiTect whole transcriptome kit showed non-linear and biased amplification of total RNA. The QuantiTect whole transcriptome kit showed no amplification in most of the samples (Ct values higher or equal to the unamplified sample) and was excluded from further evaluation.

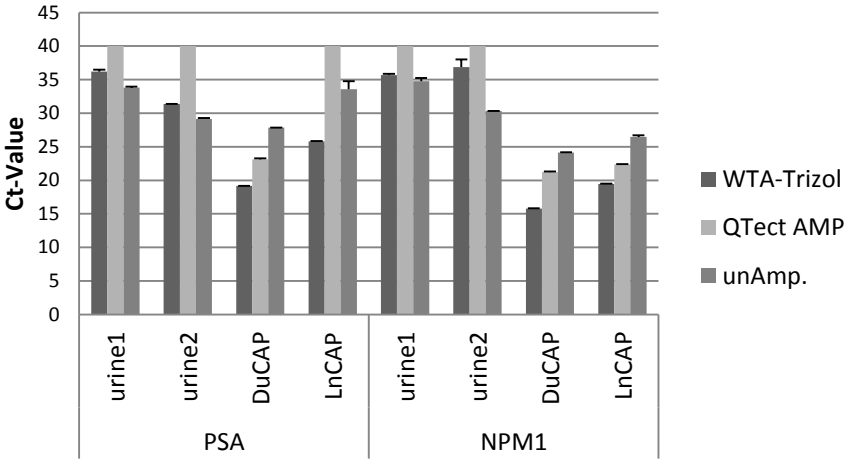



**Figure 10** Comparison of RNA amplification methods.
The expression of PSA and NPM1 genes was determined by RT-PCR in -unamplified, -WTA-amplified and -QTect-amplified RNA samples from urine and supernatants of DuCAP and LnCAP cells. WTA: whole transcriptome amplification kit from SIGMA, QTect Amp: QuantiTect whole transcriptome kit from QIAGEN.

### *PreAmp from ABI was chosen for RNA amplification*

To increase the quantity of transcripts of interest, the pre-amplification (PreAmp) kit from Applied Biosystems was also tested and compared with the WTA kit. This kit needs a pre-designed primer-pool for the genes of interest, but there is no need of DNA purification after pre-amplification. Instead, WTA amplified DNA required purification before processing for gene analysis. Two different PCR purification methods, Tri-reagent and QIAquick PCR Purification kit were applied for WTA-amplified samples. As shown in Figure 11, compared to WTA kit amplified samples, PreAmp amplified samples showed the highest and unbiased amplification of the signal and therefore this method was chosen for this study (Figure 11).

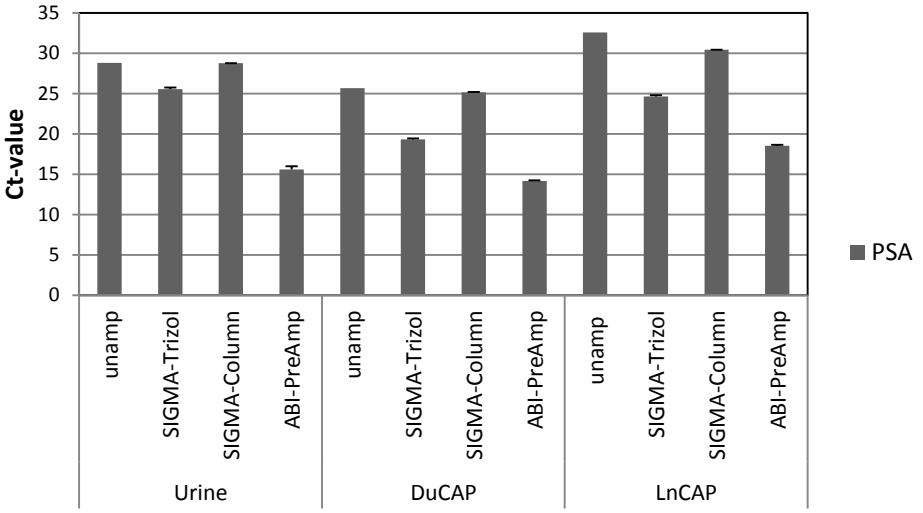



**Figure 11** The PreAmp amplification Kit was compared with the Whole transcriptome amplification (WTA) Kit for optimized amplification of RNA.
Amplification of PSA from urine exosome RNA and supernatants of DuCAP and LnCAP cell lines was tested for amplification efficiency. (unamp: unamplified, SIGMA-Trizol: amplified with SIGMA, subsequently purified using Tri-Reagent, SIGMA-Column: amplified with SIGMA, subsequently purified by QIAquick PCR Purification kit using columns, ABI-PreAmp: Preamlification kit from Applied Biosystems).

## 3.2 Analysis of potential gene mRNA markers

To investigate the expression level of the selected genes in pre-amplified RNA of urine exosomes, RT-PCR measurement were performed using a high throughput PCR system established at the MUI Sequencing and Genotyping Core Facility. In the following section the sequential analysis steps and the differences of gene expression between different groups (LGSc vs HGSc vs Be, LGSc vs Be, HSSc vs Be, LGSc vs HGSc and Ca vs Be) are described.

### 3.2.1 Sensitivity

Samples with insufficient amount of RNA or prostate-derived RNA were excluded from further analysis. Of the 60 samples 8 (5 Be, 3 LGSc) were finally excluded due to lack of housekeeping gene expression (indicating insufficient amount of RNA) and/or lack of PSA or KLK2 amplification (indicating no prostate-derived RNA) or outlier gene expression samples (indicating sample problems). In the end, 52 of 60 samples (87%), 25 benign, 10 high Gleason and 17 low Gleason were included in the analysis.

### 3.2.2 Choosing the housekeeping gene

Housekeeping genes (HKGs) are typically constitutive genes that are equally expressed in all cells under normal and pathophysiological conditions. Since the yield of isolated total RNA from urine exosomes was too little to measure the amount using classical photo-spectrometry, selection of proper HKG was important to normalize gene expression. Expression of five candidate HKGs (RHOA, HPRT1, TBP, PA2G4 and ACTB) were analyzed and compared to the average signal of all measured genes. In addition, correlations between those HKGs were investigated by *Pearson* correlation (Tables 6-7).

| Housekeeping gene candidates | Slope | PCR efficiency (%) | Ct Range |
|---|---|---|---|
| RHOA (Ras homolog family member A) | -3,31 | 100,6 | 19 - 37 |
| HPRT1 (Hypoxanthine phosphoribosyltransferase 1) | -3,57 | 90,7 | 23 - 39 |
| TBP (TATA-box binding protein) | -3,98 | 78,2 | 24 - 32 |
| ACTB (Beta Actin) | -3,46 | 94,6 | 21 - 37 |
| PA2G4 (Proliferation-associated 2G4) | -4,21 | 72,7 | 21 - 39 |

**Table 6** Houskeeping gene candidates.
The PCR-efficiency for each gene was calculated according to the equation E = -1+10(-1/slope)

**Korrelationen**

| | | | RHOA | PSA | HPRT1 | KLK2 | TBP |
|---|---|---|---|---|---|---|---|
| Spearman-Rho | RHOA | Korrelationskoeffizient | 1,000 | ,622** | ,965** | ,930** | ,579** |
| | | Sig. (2-seitig) | . | ,000 | ,000 | ,000 | ,000 |
| | | N | 59 | 53 | 47 | 54 | 41 |
| | PSA | Korrelationskoeffizient | ,622** | 1,000 | ,599** | ,582** | ,691** |
| | | Sig. (2-seitig) | ,000 | . | ,000 | ,000 | ,000 |
| | | N | 53 | 53 | 45 | 51 | 41 |
| | HPRT1 | Korrelationskoeffizient | ,965** | ,599** | 1,000 | ,930** | ,538** |
| | | Sig. (2-seitig) | ,000 | ,000 | . | ,000 | ,000 |
| | | N | 47 | 45 | 47 | 45 | 38 |
| | KLK2 | Korrelationskoeffizient | ,930** | ,582** | ,930** | 1,000 | ,530** |
| | | Sig. (2-seitig) | ,000 | ,000 | ,000 | . | ,000 |
| | | N | 54 | 51 | 45 | 54 | 40 |
| | TBP | Korrelationskoeffizient | ,579** | ,691** | ,538** | ,530** | 1,000 |
| | | Sig. (2-seitig) | ,000 | ,000 | ,000 | ,000 | . |
| | | N | 41 | 41 | 38 | 40 | 41 |

**. Die Korrelation ist auf dem 0,01 Niveau signifikant (zweiseitig).

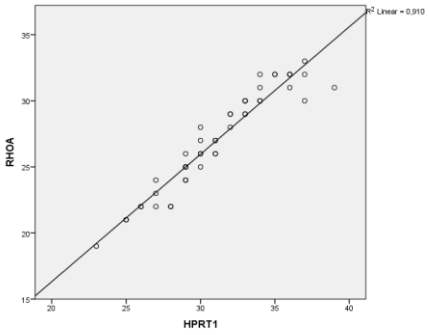



**Table 7** Correlations between housekeeping genes.
The right graph shows the correlation of RHOA and HPRT1. RHOA correlated almost perfectly with the second best HKG gene HPRT1 ($R^2$=0.965).

## *RHOA as housekeeping gene*

Of the five housekeeping genes tested (ACTB, PA2G4, TBP, RHOA, HPRT1) only RHOA showed 100% PCR efficiency (Table 6). It is the most constitutively expressed gene between groups, well correlated to the average mRNA signal ($R^2$=0.963, data not shown) and to the prostate tissue housekeeping gene HPRT1 ($R^2$=0.965) (Table 7). HPRT1 was the second best HKG, but was less abundant compared to RHOA. Therefore, RHOA was used to exclude the samples with low RNA yield (no expression of HPRT1). Furthermore, RHOA showed the best correlation to the average expression of all mRNAs between groups (Figure 12). RHOA was therefore chosen as the housekeeping gene, and the expression of other mRNAs was normalized by it.

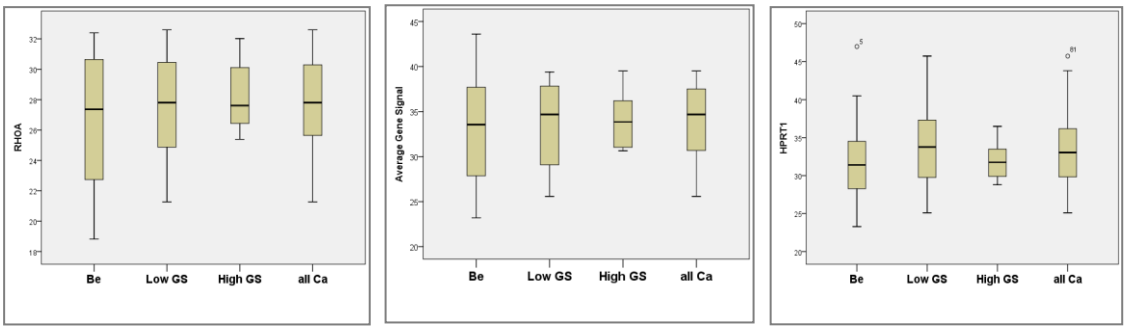



**Figure 12** Evaluation of RHOA as a housekeeping gene.
The first box-plot shows the expression level of RHOA in benign/cancer subgroups. The second diagram shows the expression level of the average of all the genes (mRNAs) examined. The third one shows the expression level of HPRT1 in all groups. RHOA showed the best uniformity between groups and similarity to the average gene signal and 100 % PCR efficiency. Therefore, RHOA was finally chosen as HKG for this study.

### 3.2.3 Discriminatory gene markers

The expression of gene marker candidates was calculated by the “ratio” formula (see 2.5: Material and Methods/Real Time PCR), and converted to the “1/ratio” values. After filtering and cleaning (as described in 2.5.3: Material/Methods, “Filtering of inconsistent data”) of the data, the data were analyzed using the SPSS analysis software package. The Kruskal-Wallis test was applied to test for the differences among groups (Be vs LGSc vs HGSc and Be vs Ca).

More than 15 markers were identified to be significantly differently expressed between subgroups (Be vs LGSc, Be vs HGSc, LGSc vs HGSc and Be vs Ca) by applying the Mann Whitney-U Test. Markers showing significant or close to significance differences are summarized in Table 8 and more detailed in Table9.

| | | | | | |
|---|---|---|---|---|---|
| Be - Ca | PCA3 | P= 0,001 * | Be - LGSc | PCA3 | P= 0,003 * |
| | PSA | P= 0,015 * | | KLK2 | P= 0,005 * |
| | KLK2 | P= 0,022 * | | TFF3 | P= 0,053 |
| | PA2G4 | P= 0,030 * | | VDAC1 | P= 0,075 |
| | MME | P= 0,058 | | | |
| | ERG | P= 0,063 | Be - HGSc | PSA | P= 0,002 * |
| LGSc - HGSc | VDAC1 | P= 0,006 * | | PA2G4 | P= 0,008 * |
| | TFF3 | P= 0,014 * | | PCA3 | P= 0,014 * |
| | HPRT1 | P= 0,031 | | ACTB | P= 0,014 * |
| | KLK2 | P= 0,035 | | ERG | P= 0,026 |
| | AGR2-Sh | P= 0,035 | | AGR2-Lt | P= 0,047 |
| | AGR2-Lt | P= 0,039 | | HPRT1 | P= 0,068 |
| | TMPRSS2 | P= 0,040 | | AMACR | P= 0,068 |
| | ACTB | P= 0,071 | | BLVRB | P= 0,068 |
| | PSA | P= 0,088 | | GOLM1 | P= 0,080 |

**Table 8** Gene marker list with significant differences or close to significance differences between different groups (Be vs Ca, …) Normalized expression data were analyzed by Kruskal-Wallis Test. Subsequently, differences found between groups were identified by the Mann Whitney U Test. P-values shown in the table are Bonferroni corrected: red colored P-values are statistically significant, P-values marked with a star remain statistically significant after Bonferroni correction for multiple testing, blue colored P-values indicate those markers with close to statistical significance (p=0,05 - 0,09). Some of the gene markers are significant in more than one comparison, e.g. PCA3, PSA, KLK2, TFF3.

Interestingly, the most powerful gene markers separating benign from cancer are three genes specifically expressed in prostate, PCA3, PSA and KLK2, in addition also PA2G4. To distinguish high grade from low grade tumors, TFF3 and VDAC1 and with some limitations also HPRT1, AGR2 and KLK2 are useful urine markers (see table 8). Of all gene markers investigated PSA, PCA3, TFF3, PA2G4, KLK2 were identified as the best discriminatory mRNA marker candidates showing significance in more than one subgroup comparisons whereas HPRT1, TMPRSS2, AGR2-Lt, AGR2-Sh and ERG showed significance in a single comparison (without Bonferroni correction). The discriminatory power of other genes including MME, BLVRB, GOLM1 and AMACR did not reach statistical significance.

| Discriminatory gene markers | Slope | PCR efficiency (%) | Ct Range |
|---|---|---|---|
| * **TFF3** (trefoil factor 3) | -3,35 | 98,9 | 21 - 38 |
| * **PCA3** (prostate cancer antigen 3) | -3,42 | 96,1 | 18 - 38 |
| * **PSA** (prostate specific antigen) | -3,18 | 106,2 | 16 - 39 |
| * **KLK2** (kallikrein 2) | -3,90 | 80,5 | 19 - 38 |
| * **PA2G4** (proliferation-associated 2G4) | -4,21 | 72,7 | 21 – 39 |
| * **ACTB** (beta Actin) | -3,46 | 94,6 | 21 - 37 |
| * **VDAC1** (voltage-dependent anion-selective channel protein 1) | -3,84 | 82,0 | 24 - 38 |
| **ERG** (ets related gene) | -3,46 | 94,5 | 29 - 36 |
| **TMPRSS2** (transmembrane protease, serine 2) | -3,81 | 82,9 | 19 - 38 |
| **HPRT1** (hypoxanthine phosphoribosyltransferase 1) | -3,57 | 90,7 | 23 - 39 |
| **AGR2-Sh** (anterior gradient homolog 2-Short transcript) | -4,68 | 63,5 | 22 - 38 |
| **AGR2-Lt** (anterior gradient homolog 2-Long transcript) | -2,41 | 159,9 | 28 - 34 |
| **BLVRB** (biliverdin reductase B) | -3,49 | 93,5 | 22 - 37 |
| **MME** (membrane metallo-endopeptidase) | -3,77 | 84,2 | 21 - 39 |
| **GOLM1** (golgi membrane protein 1) | -3,42 | 96,2 | 23 - 36 |
| **AMACR** (alpha-methylacyl-CoA racemase) | -3,95 | 79,1 | 20 - 36 |

**Table 9** Discriminatory gene markers and their PCR efficiency range.
Genes with stars are the best discriminatory mRNA marker candidates. The PCR efficiency for each gene was calculated according to the equation $E = -1+10^{(-1/slope)}$

CLU, F3, TMSB15A, FOLH1, GPR116, STEAP4, BAG1-wt, BAG1-D, GNMT, ANXA2, NPM1, PDIA3, TBP and MELK were the genes whose expression levels were not found significantly discriminant in different comparisons (Be vs Ca, Be vs LGSC, Be vs HGSC and LGSC and HGSC) in the study. (Table 10)

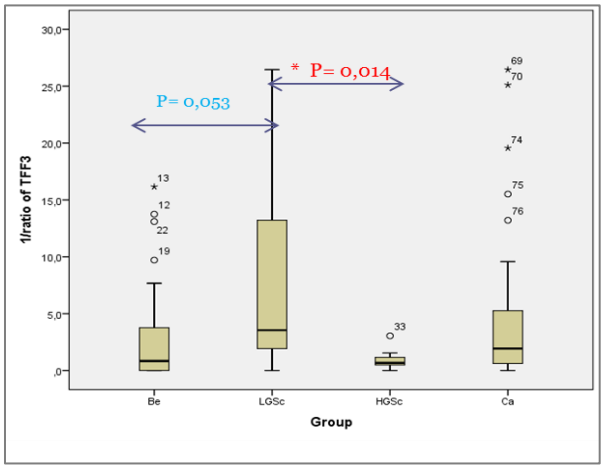
| Non-discriminatory gene markers | P-value | | Non-discriminatory gene markers | P-value |
|---|---|---|---|---|
| GNMT | 0,178 | | FOLH1 | 0,602 |
| PDIA3 | 0,210 | | GPR116 | 0,640 |
| TMSB15A | 0,241 | | STEAP4 | 0,707 |
| BAG1-wt | 0,268 | | CLU | 0,707 |
| F3 | 0,374 | | MELK | 0,833 |
| BAG1-D | 0,394 | | NPM1 | 0,891 |
| ANXA2 | 0,458 | | TBP | 0,920 |
| TMPRSS2-ERG (fusion gene) | * | | | |

**Table 10** Gene markers neither significant nor close to significance are listed.
*TMPRSS2-ERG was not added to statistical analyses due to low detection rate.

**Group-specific classification of gene markers:** PA2G4, ACTB and MME were found to be increased in Benign urine samples compared to Cancer, particularly compared to HGSc. TFF3, PCA3, KLK2, TMPRSS2 and both AGR2 transcript variants revealed an up-regulation in low-Gleason compared to high-Gleason cancer, whereas PSA, ERG, HPRT1, VDAC1, BLVRB, AMACR and GOLM1 were more expressed in high-Gleason tumors of PCa patients compared to Benign and LGSc cancer. This pattern suggests that more cancer cells and exosomes were released into the urine from prostate tumor and especially high grade tumors. This could be attributed to the specific properties of tumor cells and/or the histoarchitectural alterations in advanced diseases tissue.

### *TFF3 (trefoil factor 3)*

TFF3, a member of trefoil factor family, has been involved in many types of cancer and inflammatory diseases. Its mRNA level revealed discriminatory power between the cancer subgroups LGSc and HGSc (P=0.014; confirmed by Bonferroni method), whereas the difference between benign and LGSc was almost statistically significant. (P=0.053) (Figure 13). Thus TFF3 can be considered as a potential marker to distinguish LGSc and HGSc tumors in prostate cancer patients.

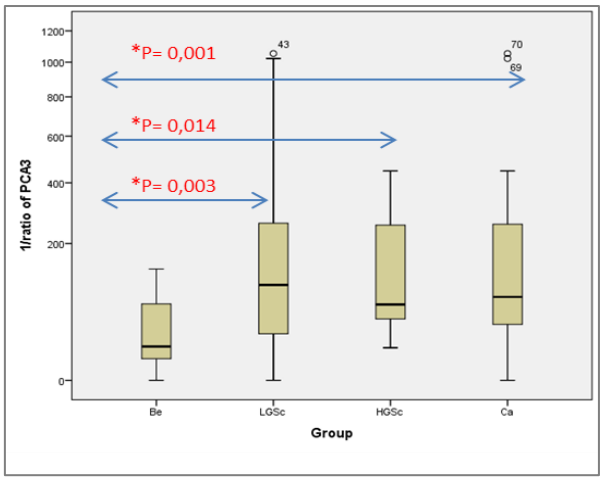



| Ranges | | | | |
|---|---|---|---|---|
| Group | | N | Middle Rang | Total Rang |
| TFF3 | LGSc | 17 | 16,88 | 287,00 |
| | HGSc | 10 | 9,10 | 91,00 |
| | Total | 27 | | |
| TFF3 | Be | 25 | 18,48 | 462,00 |
| | LGSc | 17 | 25,94 | 441,00 |
| | Total | 42 | | |

**Figure 13** Expression pattern of TFF3 mRNA in urinary exosomes.
TFF3 expression was analyzed by RT-PCR in 30 benign and 30 cancer urine samples of prostate cancer (PCa) patients collected after DRE (digital rectal examination) and stored frozen for 5-7 years. Exosomes from urine were isolated by ultrafiltration, total RNA was purified by Direct-zol MiniPrep kit from Zymoresearch. RT-PCR was run in quadruplicate and the relative expression ratio of the gene was computed based on its PCR-efficiency and $C_t$ -value difference to the house keeping gene RHOA using the Pfaffl method (124). The diagram shows the relative transcription levels of 25 benign (Be), 27 cancer (Ca) (17 low Gleason (LGSc) = GSc ≤ (3+4) and 10 high Gleason (HGSc) = GSc ≥ (4+3) urine samples. 8 samples including outliers had to be excluded. The data are shown as a box blot. Lines indicate the median values. In addition, the middle range and p-values calculated by the Mann-Whitney-Test are indicated. Red p-values indicate statistically significant differences, blue p-values differences close to statistical significance. A star (*) marks those differences that remain statistically significant after Bonferroni correction for multiple testing.

### *PCA3 (prostate cancer antigen 3)*

This prostate-specific marker has been discovered in 1999 and was found as a prostate-specific gene that is highly overexpressed in prostatic tumor tissues (55). In line with this pattern, in our urinary exosomes samples PCA3 was significantly higher expressed in cancer compared with benign samples (Figure 14). When the cancer samples were further divided into LGSc and HGSc, both subgroups showed higher expression compared to benign samples, although no difference was seen between LGSc and HGSc cancer samples of patients. All 3 differences stayed significant after Bonferroni correction.

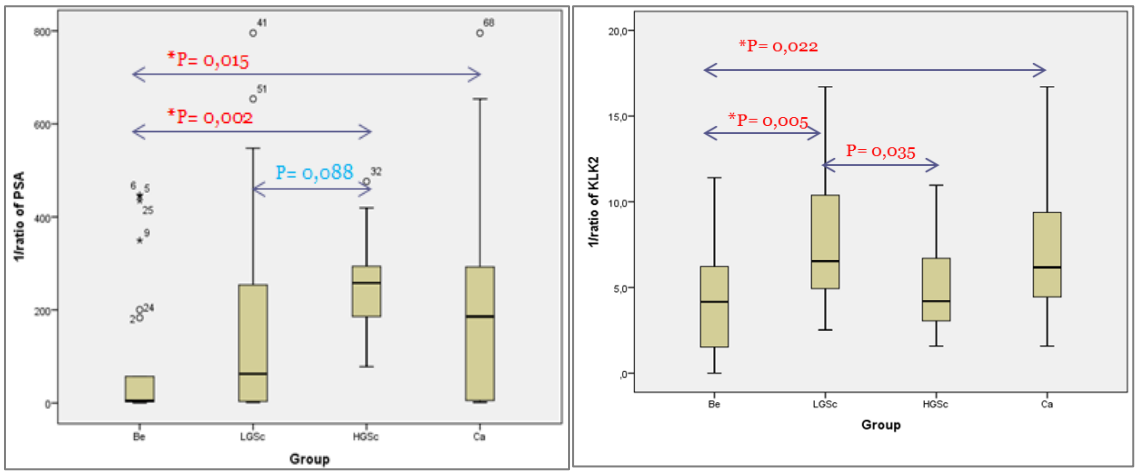



| Ranges | | | | |
|---|---|---|---|---|
| Group | | N | Middle Rang | Total Rang |
| PCA3 | Be | 25 | 16,84 | 421,00 |
| | LGSc | 17 | 28,35 | 482,00 |
| | Total | 42 | | |
| PCA3 | Be | 25 | 15,32 | 383,00 |
| | HGSc | 10 | 24,70 | 247,00 |
| | Total | 35 | | |
| PCA3 | Be | 25 | 19,16 | 479,00 |
| | Ca | 27 | 33,30 | 899,00 |
| | Total | 52 | | |

**Figure 14** Expression pattern of PCA3 mRNA in urinary exosomes.
PCA3 expression was determined and analyzed according to the procedure described in the legend to Figure 13.

### *PSA (prostate specific antigen) and KLK2 (kallikrein-2)*

KLK2 and PSA alias KLK3 are both serine proteases and belongs to the Kallikrein gene family. Measurement of KLK2 was less efficient (80%) compared to PSA (106%) in RT-PCR but both genes were found higher expressed in cancer and showed good differences between groups. Whereas PSA was highly expressed in high Gleason KLK2 showed overexpression in low Gleason tumors (Figure 15). PSA could distinguish benign from cancer, particularly benign from high Gleason samples of PCa patients. The difference between low and high Gleason cancer groups reached almost statistical significance.
KLK2 could differentiate benign from cancer and from low Gleason tumor, while a difference was also seen between LGSc and HGSc tumor samples of PCa patients.

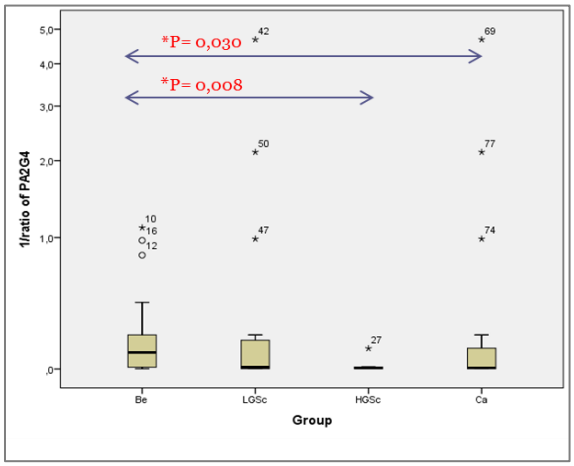



| Ranges | | | | | Ranges | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Group | | N | Middle Rang | Total Rang | Group | | N | Middle Rang | Total Rang |
| PSA | Be | 25 | 14,64 | 366,00 | KLK2 | Be | 25 | 17,08 | 427,00 |
| | HGSc | 10 | 26,40 | 264,00 | | LGSc | 17 | 28,00 | 476,00 |
| | Total | 35 | | | | Total | 42 | | |
| PSA | Be | 25 | 21,20 | 530,00 | KLK2 | Be | 25 | 21,48 | 537,00 |
| | Ca | 27 | 31,41 | 848,00 | | Ca | 27 | 31,15 | 841,00 |
| | Total | 52 | | | | Total | 52 | | |
| PSA | LGSc | 17 | 12,00 | 204,00 | KLK2 | LGSc | 17 | 16,47 | 280,00 |
| | HGSc | 10 | 17,40 | 174,00 | | HGSc | 10 | 9,80 | 98,00 |
| | Total | 27 | | | | Total | 27 | | |

**Figure 15** Expression pattern of PSA and KLK2 mRNA in urinary exosomes.
PSA and KLK2 expression was determined and analyzed according to the procedure described in the legend to Figure 13.

### *PA2G4 (proliferation-associated 2G4)*

PA2G4 is one of the genes initially considered as housekeeping gene candidates in the study. However, it turned out to be differentially expressed. It is overexpressed in the benign compared to the cancer group (see ranges in Figure 16). It also significantly distinguished high Gleason tumors from benign samples of PCa patients.

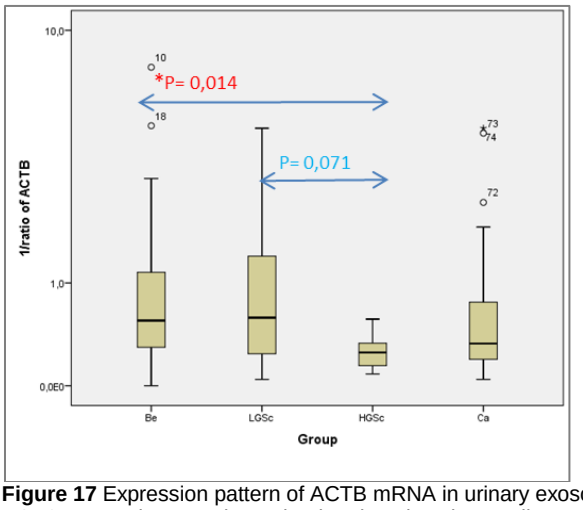



| Ranges | | | | |
|---|---|---|---|---|
| Group | | N | Middle Rang | Total Rang |
| PA2G4 | Be | 25 | 20,92 | 523,00 |
| | HGSc | 10 | 10,70 | 107,00 |
| | Total | 35 | | |
| PA2G4 | Be | 25 | 31,24 | 781,00 |
| | Ca | 27 | 22,11 | 597,00 |
| | Total | 52 | | |

**Figure 16** Expression pattern of PA2G4 mRNA in urinary exosomes.
PA2G4 expression was determined and analyzed according to the procedure described in the legend to Figure 13.

### *ACTB (beta actin)*

ACTB is a commonly used reference gene for many expression studies and was also chosen as housekeeping gene candidate for our study. However, ACTB is higher expressed in benign samples compared to the high Gleason tumor samples, although no difference was seen between benign and low Gleason samples of PCa patients (Figure 17). Moreover, it also showed almost significant difference between the LGSc and HGSc cancer subgroups.

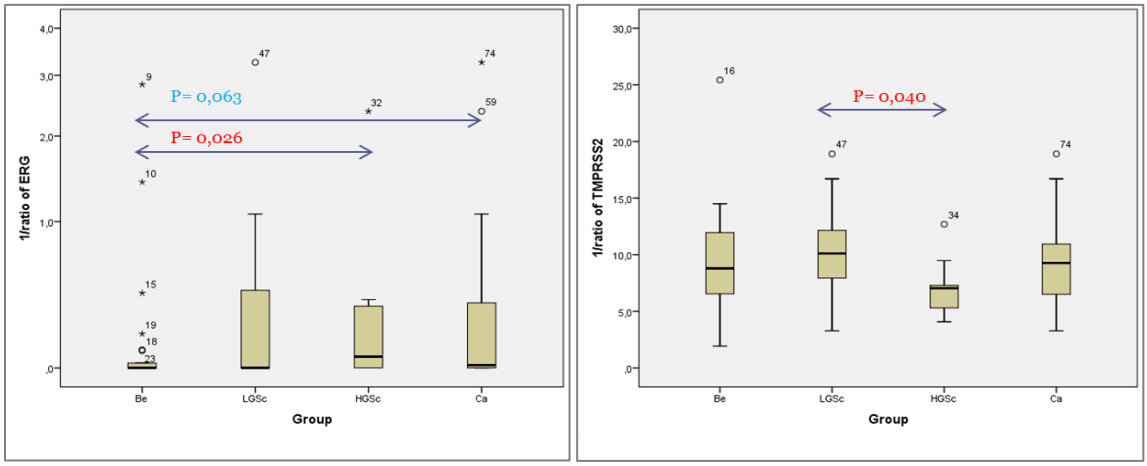



| Ranges | | | | |
|---|---|---|---|---|
| Group | | N | Middle Rang | Total Rang |
| ACTB | Be | 25 | 20,68 | 517,00 |
| | HGSc | 10 | 11,30 | 113,00 |
| | Total | 35 | | |
| ACTB | LGSc | 17 | 16,12 | 274,00 |
| | HGSc | 10 | 10,40 | 104,00 |
| | Total | 27 | | |

**Figure 17** Expression pattern of ACTB mRNA in urinary exosomes.
PCA3 expression was determined and analyzed according to the procedure described in the legend to Figure 13.

### *TMPRSS2, ERG and TMPRSS2-ERG fusion gene mRNAs*

TMPRSS2 (transmembrane protease, serine 2) is an androgen regulated gene, ERG (ets related gene) a transcription factor of the ETS family. In 50-70% of prostate cancer tumors there is a fusion of these two genes, which entails transcriptional control of ERG expression by the androgen receptor. This results in a specific up-regulation of ERG expression. The real-time PCR assay for the TMPRSS2 gene used in this study determines the wild-type TMPRSS2 gene only, whereas the ERG assay measures both the normal ERG mRNAs and the TMPRSS2-ERG fusion gene mRNA. Whereas expression of the ERG transcript showed differences between benign and tumor, especially high grade tumor samples of patients, TMPRSS2 showed the highest expression in LGSc and a significant difference between LGSc and HGSc tumor of PCa patients urine (Figure 18).




| Ranges | | | | |
|---|---|---|---|---|
| Group | | N | Middle Rang | Total Rang |
| ERG | Be | 25 | 22,44 | 561,00 |
| | Ca | 27 | 30,26 | 817,00 |
| | Total | 52 | | |
| ERG | Be | 25 | 15,56 | 389,00 |
| | HGSc | 10 | 24,10 | 241,00 |
| | Total | 35 | | |
| TMPRSS2 | LGSc | 17 | 16,41 | 279,00 |
| | HGSc | 10 | 9,90 | 99,00 |
| | Total | 27 | | |

**Figure 18** Expression pattern of ERG and TMPRSS2 mRNA in urinary exosomes.
ERG and TMPRSS2 expression was determined and analyzed according to the procedure described in the legend to Figure 13.

### *TMPRSS2-ERG status*

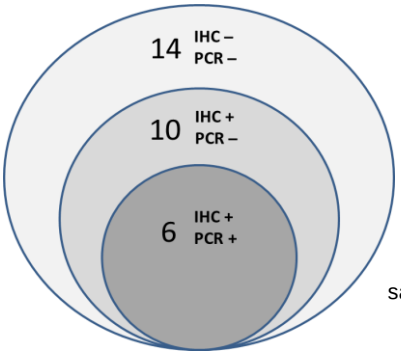


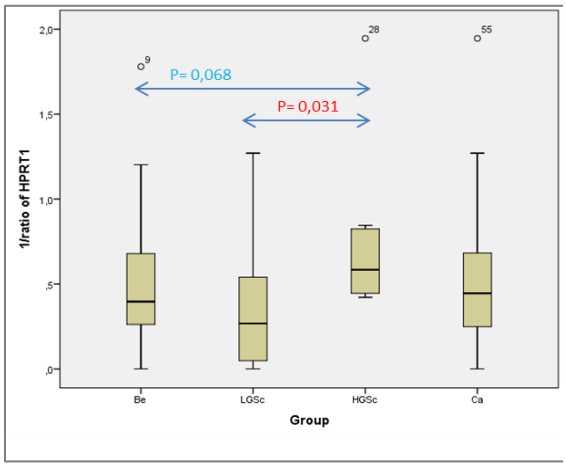

**Figure 19** Correlation of the TMPRSS2-ERG fusion status determined by immunohistochemistry (IHC) in the radical prostatectomy specimens and the exosomes TMPRSS2-ERG real-time PCR. Comparison revealed 6 fusion-positive in urine samples (PCR+), which have been confirmed by IHC analysis (IHC+); 10 samples were fusion positive in IHC but not in urine study, whereas rest 14 samples showed no ERG-rearrangement.

Of all tumor patients' samples in the study only 6 revealed a positive RT-PCR signal for the TMPRSS2-ERG fusion transcript. A number of studies revealed that the fusion of the TMPRSS2 and ERG genes indicate the existence of prostate cancer. A TMPRSS2-ERG fusion gene is found in around 50% of the prostate tumors. In this study 6 out of 27 urine exosomal RNAs from PCa patients were found ERG gene rearrangement positive, while immunohistochemistry (IHC) analysis of the radical prostatectomy tissue specimen of the same patients revealed 16 of the 27 cancer cases harboring the rearrangement (Figure 19). This indicates that analysis of urine exosome RNA can detect only a small percentage of fusion gene positive tumors in this patient cohort.

In this study the TMPRSS2:ERG fusion transcript has also been detected in 2 of 25 benign samples. One of these two patients had a tumor-negative biopsy in 2007, but was recently (2012) diagnosed with a TMPRSS2-ERG rearrangement positive adenocarcinoma, indicating that fusion gene detection in urinary exosomes can predict cancer earlier in some cases. A follow-up of the second case was not available.

### *HPRT1 (hypoxanthine phosphoribosyltransferase 1)*

HPRT1 is normally used as a HKG for analysis of expression in prostate tumor tissue and cell lines. In this exosome study we found it differentially expressed in different groups, particularly it was over-expressed in HGSc tumors. In consequence it showed a surprisingly significant difference of expression between LGSc and HGSc cancer subgroups and an almost significance difference between benign and HG tumor samples of PCa patients (Figure 20).

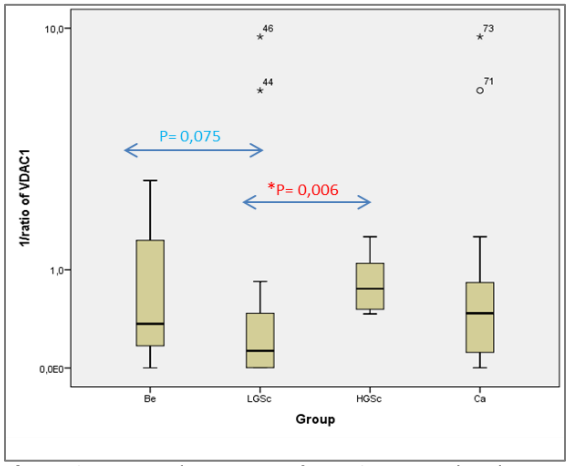



| Ranges | | | | |
|---|---|---|---|---|
| Group | | N | Middle Rang | Total Rang |
| HPRT1 | Be | 25 | 16,00 | 400,00 |
| | HGSc | 10 | 23,00 | 230,00 |
| | Total | 35 | | |
| HPRT1 | LGSc | 17 | 11,47 | 195,00 |
| | HGSc | 10 | 18,30 | 183,00 |
| | Total | 27 | | |

**Figure 20** Expression pattern of HPRT1 mRNA in urinary exosomes**.**
HPRT1 expression was determined and analyzed according to the procedure described in the legend to Figure 13.

### *VDAC1 (voltage-dependent anion-selective channel protein 1)*

As an important metabolite transporter VDAC1 is indispensable for cell functions such as energy supply or cell growth. Consistent with its dysregulation in many type of cancers (126), our study also confirmed its aberrant expression in prostate cancer (Figure 21). Statistically significant higher expression of VDAC1 was measured in the more aggressive (HGSc) compared to low Gleason (LGSc) cancer samples. But interestingly, if we focus on benign and LGSc tumor samples of PCa patients, VDAC1 expression was decreased in LGSc cancer at an almost statistically significant level (Figure 21).

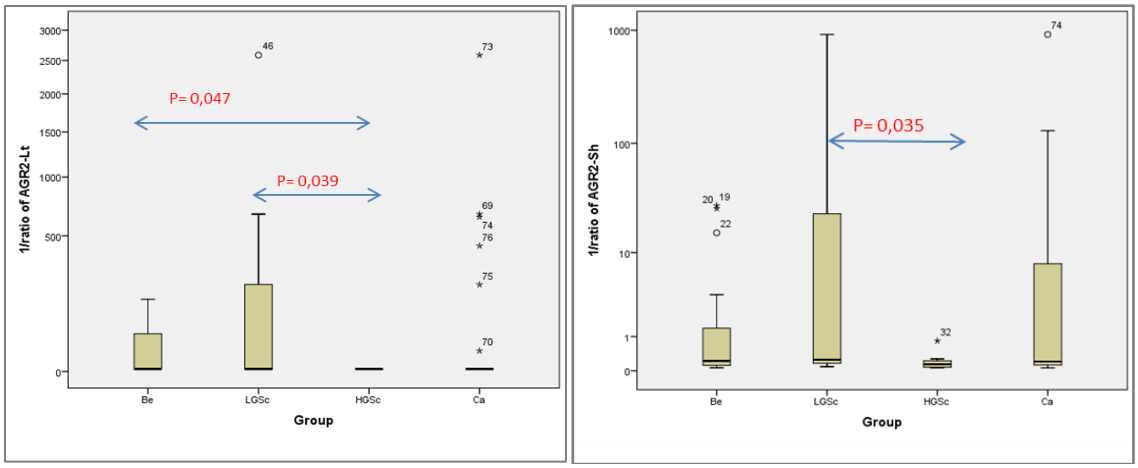



| Ranges | | | | |
|---|---|---|---|---|
| Group | | N | Middle Rang | Total Rang |
| VDAC1 | Be | 25 | 24,28 | 607,00 |
| | LGSc | 17 | 17,41 | 296,00 |
| | Total | 42 | | |
| VDAC1 | LGSc | 17 | 10,76 | 183,00 |
| | HGSc | 10 | 19,50 | 195,00 |
| | Total | 27 | | |

**Figure 21** Expression pattern of VDAC1 mRNA in urinary exosomes.
VDAC1 expression was determined and analyzed according to the procedure described in the legend to Figure 13.

### *AGR2 (anterior gradient homolog 2) variants*

Anterior Gradient 2 (AGR2) was found in mucus-secreting cells and endocrine organs and thus implicated with an epithelial barrier function and associated with inflammatory diseases (127). It is an androgen-regulated gene (128, 129). The existence of additional 5' exons has led to two different splice variants: AGR2-long transcript (AGR2-Lt) and AGR2-short transcript (AGR2-Sh). Our result showed that both AGR2 variants were higher expressed in LGSc compared to HGSc tumor samples of PCa patients, thus displaying some distinction between the two cancer subgroups (Figure 22). In addition, AGR2-Lt also exhibited a significant difference between the benign and HGSc groups.

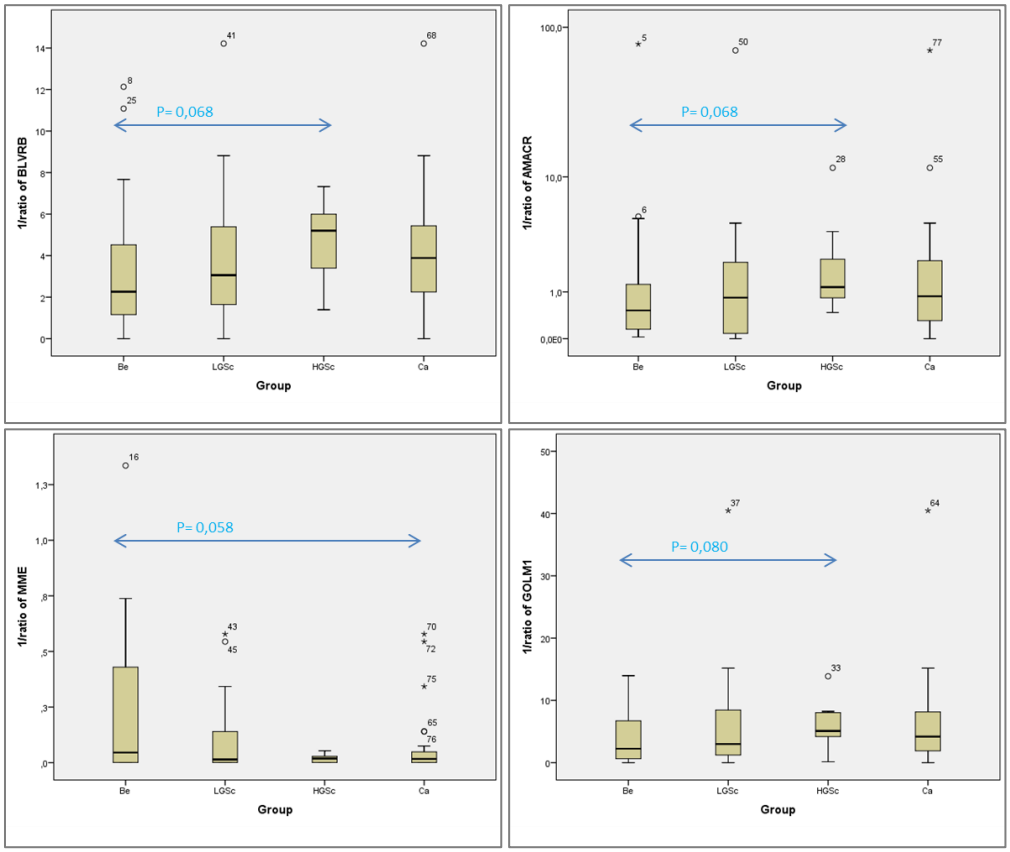



| Ranges | | | | |
|---|---|---|---|---|
| Group | | N | Middle Rang | Total Rang |
| AGR2-Sh | LGSc | 17 | 16,47 | 280,00 |
| | HGSc | 10 | 9,80 | 98,00 |
| | Total | 27 | | |
| AGR2-Lt | LGSc | 17 | 15,76 | 268,00 |
| | HGSc | 10 | 11,00 | 110,00 |
| | Total | 27 | | |
| AGR2-Lt | Be | 25 | 19,60 | 490,00 |
| | HGSc | 10 | 14,00 | 140,00 |
| | Total | 35 | | |

**Figure 22** Expression pattern of AGR2-Lt (long transcript) and AGR2-Sh (short transcript) mRNA in urinary exosomes.
The expression of the two AGR2 transcripts was determined and analyzed according to the procedure described in the legend to Figure 13.

### *Additional genes with almost statistically significant expression differences*

In addition to the genes described above, which showed significantly different expression in Be vs Ca, or between subgroups, 4 other genes, AMACR, BLVRB, GOLM1 and MME showed differences close to statistical significance (Figure 23). Except for MME, all other 3 genes showed a higher expression in the HGSc tumor compared to the benign group of PCa patients. MME was higher expressed in the benign compared to the cancer group.




| Ranges | | | | |
|---|---|---|---|---|
| Group | | N | Middle Rang | Total Rang |
| BLVRB | Be | 25 | 16,00 | 400,00 |
| | HGSc | 10 | 23,00 | 230,00 |
| | Total | 35 | | |
| AMACR | Be | 25 | 16,00 | 400,00 |
| | HGSc | 10 | 23,00 | 230,00 |
| | Total | 35 | | |
| MME | Be | 25 | 19,64 | 491,00 |
| | HGSc | 10 | 13,90 | 139,00 |
| | Total | 35 | | |
| GOLM1 | Be | 25 | 16,08 | 402,00 |
| | HGSc | 10 | 22,80 | 228,00 |
| | Total | 35 | | |

**Figure 23** Expression pattern of BLVRB (biliverdin reductase B), MME (membrane metallo-endopeptidase), GOLM1 (golgi membrane protein 1), AMACR (alpha-methylacyl-CoA racemase) mRNA in urinary exosomes.
The expression of these genes was determined and analyzed according to the procedure described in the legend to Figure 13.

# 4 DISCUSSION

Prostate cancer (PCa) is the most frequently diagnosed cancer in European men, the sixth most common cancer in the whole world. Early diagnosis has substantial importance for the treatment of PCa. Despite of the prevalent use of PSA as a screening marker, its deficiency in specificity and lack of prognosis values promotes the discovery of new biomarker or combination of biomarkers that add further information to clinical and pathological analysis. Potential PCa biomarkers can be present in body fluids released from malignant tissue. As a body fluid urine is an important source of biomarkers due to availability, easy collection and usability in clinical diagnosis. Urine is also a reservoir of bioactive vesicles, such as exosomes, which can be used for diagnostic approaches (130). Recently, urine has been studied for markers of various kinds of cancer, including gastric, renal, ovarian, epididymal, bladder, breast and prostate cancer diseases (131-136). Lately, urinary matrix proteins have been found even to predict the presence of brain tumors (137).

Tissue-derived microvesicles known as exosomes are present in bodily fluids including serum and urine. They are secreted by normal or tumor cells and contain various molecular constituents of the cell of origin including proteins and functional RNA (110). Exosomes can concentrate specific RNA (more than 60 times) compared to that directly isolated form from blood, serum or plasma (138, 139) and can serve as biomarkers for cancer diagnosis and prognosis. Moreover, in comparison to urine sediments, the RNA integrity profile is better conserved in urinary exosomes suggesting that microvesicles may protect RNA from degradation (140, 141). With this assumption that potential disease biomarker transcripts may be identifiable in urinary exosomes, we have carried out a multiple gene expression analysis of RNAs isolated from urine exosomes. Consistent with previous studies, in this study we were able to isolate RNA from urine samples that were kept at -80°C for 5 years, suggesting frozen long-term storage did not harm the quality and retrieval of RNA from urinary exosomes (142).

Exosomal RNA instead of protein analysis was chosen in this study. First of all, several proteomics analyses have been carried out for urine exosomes, while urinay exosome RNA analyses to our knowledge are very scarce. Secondly, we are limited by the equipment and methods required for a proteomics analysis. Thirdly, RNA has the advantage that pre-amplification of genes of interest to improve the detection sensitivity is possible. In particular, proteomic analyses require complex strategies due to competition between low and high-abundant proteins, and post-translationally modified proteins profiling is a big challenge as well (143). Proteomics profiling requires an extremely well-defined patient population, rigorous protocols of sample collection and an appropriate control group (144). The

quantification is also a major challenge, using e.g. creatinine for the normalization of protein candidates might be inadequate due to high individual variability in its rate of excretion (145). Moreover, contamination is a limiting factor, which affects the obtainable information and reduces the sensitivity by competition with exosomal proteins (139, 146). For the future, a combined RNA/Proteomics analysis would provide the best starting point to identify exosomal disease biomarkers (147).

Several methods were reported to isolate exosomes from body fluids: ultrafiltration, immunoaffinity purification and ultracentrifugation with precipitation or density gradient flotation. Differential ultracentrifugation is effective for samples up to 250ml, but it requires expensive instrumentation and long processing times, and, it is also unpractical for a large number of samples (145, 148). Ultrafiltration for exosome RNA isolation is preferred in some studies to circumvent the limitation of instrument accessibility (149, 150). For characterization of the isolated exosomes, electron microscopy, flow cytometry and western blot methods were used to analyze their morphology and protein content (139). None of these methods are appropriate to identify tissue-specific exosomes. In this study, we used ultrafiltration for exosome isolation, as a previous pilot test showed similar yield of RNA compared to ultracentrifugation (data not shown). However, the limitation of this method is the purity of exosomes components, as large protein complexes may be cofiltered together with exosomes. Moreover, this method yielded not prostate tissue-specific exosomes, but a heterogenous population of microvesicles sheded from different tissues. Therefore, immunoaffinity purification using antibody-coated beads or columns targeting tissue-specific membrane proteins is a future direction to isolate more pure exosomes derived from the specific tissue type of interest (151). Despite that many of the PCa markers, e.g., AMACR, ERG, VDAC1 and TFF3, are also expressed by non-prostatic cell types, they were found to be significantly higher expressed in the urine exosomes from PCa patients in our study. On the other side, some genes, like MELK, PDIA3, NPM1, FOLH1, GNMT that were reported to be markers for PCa tissue, were not found significantly different in the study. This may be due to the broad tissue expression of these genes, e.g. also in other urinary tract organs, which feed their exosomes into the urine. This lack of specificity may show the requirement of highly sensitive strategies to improve detection of tissue-specific exosomes. Further investigations on exosomal surface proteins may provide a solution of this problem in the future.

One focus of this study was to establish a valid protocol in order to isolate and preamplify RNA from urine exosomes and to proof the feasibility of quantification of a mRNA marker panel in urinary exosomes. After the comparison of 3 different RNA isolation methods (manual TRI-reagent isolation, Trizol RNA Purification Kit from Zymo Research, and a

vacuum technique by QIAamp Circulating Nucleic Acid Kit), we have chosen the nucleic acid adsorption membrane column based Kit from Zymo Research due to easy handling, low risk of nucleic acid loss and its capacity for handling large scale samples. Manual isolation by the TRI-Reagent method was inconvenient, and in addition there was a risk of RNA loss during the washing steps. Recent studies also indicate that column-based methods end up with the highest RNA yield and broadest RNA size distribution, whereas phenol and combined phenol-column based approaches lost primarily large RNAs and captured a reduced yield of exosomal RNA (152).

Even with an optimized RNA isolation technique the amount from urinary exosome samples is too low for a multiplex real-time PCR analysis. Amplification techniques have to be used to further increase the amount of starting material. For the amplification of total RNA we tested 3 amplification methods (WTA kit from SIGMA, QuantiTect kit from QIAGEN and TaqMan Kit from ABI). The QuantiTect kit from QIAGEN was excluded due to impracticalness for amplifying potentially (partially) degraded exosomal RNA. Moreover, the WTA kit from SIGMA needed more tedious work and showed biased amplification and was finally also excluded. The PreAmp Kit from ABI showed un-biased amplification and high enrichment of genes of interest and was therefore chosen for this study. The same procedure was also used in the serum microvesicle study of Noerholm *et al* (153).

All 32 mRNA transcripts were analyzed by RT-PCR (qPCR) using pre-amplified samples. Generally, quantification of mRNA can be normalized by different methods like against cell number, total RNA mass or expression level of a housekeeping gene. We determined the levels of expressed genes by applying a “relative quantification strategy”, which was based on the expression levels of a target gene versus a reference (or housekeeping) gene, as proposed by Pfaffl (124). The choice of a housekeeping gene (HKG) for exosome RNAs remained a challenge, as not much is known about the expression profiles of exosomes. In several similar studies different reference genes were used to normalize exosomal RNA quantity including GAPDH, which was the mostly used, and also 18S rRNA, ß-actin (ACTB) and CD24 (147, 153-155). In our study, we tested the consistent expression of 5 potential reference genes (RHOA, HPRT1, PA2G4, ACTB, TBP). Some of these HKG candidates (HPRT1, ACTB, TBP) have been used to normalize mRNA from tissue samples, whereas RHOA and PA2G4 were included due to their high expression in PCa cell lines in previous microarray studies (data not shown). Interestingly, HPRT1 (LGSc-HGSc), PA2G4 (Be-Ca and Be-HGSc) and ACTB (Be-HGSc and LGSc-HGSc) showed statistically significant differences between groups. Ras homolog gene family member A (RHOA) is a small GTPase regulating the actin cytoskeleton and the remodeling of cell shape and orientation

(156). It showed the least differences between the analyzed groups and was therefore chosen as the reference gene for normalization in this study.

Using high through-put RT-PCR technology provided by the DNA sequencing and genotyping core facility unit of the Innsbruck Medical University, we analyzed more than 30 mRNA transcripts in 60 urinary exosome RNAs. In view of quadruplicate measurements and different controls included in the RT-PCR analysis around 7500 single PCR reactions were performed, which illustrates the importance of an appropriate automatization technique and appropriate data processing pipeline for statistical analysis. We have identified 7 single genes showing best discriminatory power even after Bonferroni correction for multiple testing: TFF3 (Be-LG, LG-HG), PCA3 (Be-Ca, Be-LG, Be-HG), ACTB (Be-HG, LG-HG) and PA2G4 (Be-Ca, Be-HG) as described in the Results section, PSA (Be-Ca, Be-HG, LG-HG), KLK2 (Be-Ca, LG-HG), VDAC1 (LG-HG, Be-LG). In addition, TMPRSS2 (LG-HG), AGR2-Lt (LG-HG, Be-HG), AGR2-Sh (LG-HG), ERG (Be-HG) also revealed significant differences, but only without Bonferroni correction.

Among the top discriminatory genes three prostate-specific genes come forward: PSA, KLK2 and PCA3. In contrast, the other 4 genes, TFF3, ACTB, PA2G4 and VDAC1 are expressed in significant amounts in non-prostate tissues as well. KLKs belong to the chymotrypsin family of serine proteases, originally identified in pancreas and named after this organ (kallikreas) in Greek (157). Prostate specific antigen (PSA) and the same family member human kallikrein 2 (KLK2) share 80% sequence homology and are both primarily expressed in the prostate gland, but they differ in their enzymatic activity. In our study PSA was well detected in the majority of urinary exosomes, its abundance was higher than that of KLK2. This is consistent with another study that showed the levels of KLK2 mRNA transcript to be about half of that of the PSA transcript (158). Both genes could significantly discriminate benign from cancer. Particularly, PSA was overexpressed in high-grade tumors compared to other groups. It discriminated Be vs HGSc (P=0.002) and Be vs Ca (P=0.015) (both p-values Bonferroni corrected), and almost significantly LGSc vs HGSc (P=0.088). In accordance with these results, PSA mRNA in urine is found higher in men diagnosed with PCa when compared to men without oncologic disease (159). On the other hand, KLK2 was found expressed predominantly in low-grade cancer in our study. It distinguished benign from LGSc (P=0.005) and from cancer (P=0.022) (Bonferroni corrected). Further it differentiated LGSc from HGSc (P=0.035) (without Bonferroni correction). Vickers and colleagues suggested the combination of KLK2 protein with PSA isoforms (total, free and intact) in blood to improve the prediction of prostate biopsy results in men with elevated PSA (160).

Hypoxanthine phosphoribosyltransferase 1 (HPRT1) is a transferase playing a central role in the purin salvage pathway and its deficiency resulted in many diseases including urinary tract disorders (161). It has been preferred as an internal reference gene in many studies to normalize gene expression in tissue samples (162-166). Although HPRT1 is a good HKG for prostate tissue, it was not suitable for urinary exosome RNAs, as it was not present at a high level in urine samples and was undetectable in 20% of all samples (6 Be, 6 LGSc). Moreover, we have found HPRT1 elevated in HGSc tumor samples when compared to LGSc tumors (P=0.031), while showing also almost significant difference between Be and HGSc tumor samples (P=0.068). HPRT1 was also reported unsuitable as a HKG in blood sample RNA (167).

Proliferation-associated-2G4 (PA2G4), also known as ErbB3 binding protein 1 (EBP1), is involved in growth regulation and was implicated in PCa progression. As a transcription factor it plays a role in androgen receptor gene regulation (168). In our study, PA2G4 was found to be highly expressed in benign samples, thus distinguishing Be vs Ca (P=0.030) and Be vs HGSc (P=0.008), both p-values Bonferroni corrected. Recently, its metastasis-inhibiting ability was reported (169), and it was found to repress the expression of anterior gradient protein 2 (AGR2), a metastasis-promoting gene in PCa (170).

Beta-actin (ACTB) as a component of the cytoskeleton has been widely used as a reference gene/protein for quantifying expression levels in tumors. We have found ACTB less expressed in more aggressive tumors (HGSc) compared to other groups. ACTB was significantly lower expressed in HGSc compared to benign (P=0.014, Bonferroni corrected) and almost significantly different between LGSc and HGSc (P=0.071). Conversely, it has been reported that ACTB is generally up-regulated in the majority of tumor cells and tissues, and it was suggested that its overexpression promotes changes of the cytoskeleton that are related to invasiveness and metastasis of cancers (171).

Trefoil factor 3 (TFF3) is a mucus-associated peptide (172). It belongs to the TFF peptides family, which is related to both inflammatory diseases and different types of cancer. It was suggested as a prognosis marker in gastric carcinoma (173). A gene expression analysis in a tissue microarray study revealed TFF3 overexpression in primary and metastatic PCa (174), while other IHC tissue studies reported no correlation with Gleason grade, tumor stage or recurrence of PCa (175). In addition, TFF3 was also found in the plasma of patients with PCa and associated with aggressive disease (176). Our gene expression analysis in urinary exosomes confirmed association of TFF3 with PCa. We have found that TFF3 was expressed specially in samples derived from low Gleason tumor and that it could distinguish

LGSc tumor from HGSc (P=0.014, Bonferroni corrected). Moreover, its expression was higher in LGSc compared to Benign at an almost significant level (P=0.053). Conversely, TFF3 did not show expression differences in a urine sediment study (64). TFF3 has been reported differentially expressed in prostate tumors dependent on the presence or absence of a TMPRSS2-ERG gene rearrangement (see below for more detail) (177). Since we have found only a limited number of ERG fusion gene positive samples in our urine study (6 cases), we could not correlate the expression of TFF3 to TMPRSS2-ERG levels.

Prostate cancer antigen 3 (PCA3), also called DD3 is a PCa specific gene overexpressed in 95% of primary PCa specimens (54). It is a noncoding RNA with no protein product. A detection assay using the RT-PCR technique in urinary sediment samples was established and is approved for diagnostic testing for men with still high PSA serum levels despite a tumor-negative prostate biopsy (178). The PCA3 assay (uPM3 diagnostic test, Progensa® PCA3 assay from Gen-Probe) uses transcription-mediated amplification of urine sample sediments obtained after digital rectal examination (DRE). A PCA3 score is generated by normalizing the PCA3 mRNA expression to PSA. This test has the potential to improve the diagnostic specificity of PSA in selected patients, although differences in DRE may limit extensive reproducibility of this method (178). In several studies it has been reported that PCA3 seems to be superior to PSA and percent of free PSA (179, 180). Moreover, it might be considered to predict tumor aggressiveness when using urine sediments instead of whole urine samples (181). Our study is one of the very few studies to analyze PCA3 in urine exosomes. Consistent with urine sediments analysis, we observed abundant expression in urine exosomes (undetectable only in 2 samples; 1 Be, 1 Ca). A significantly higher PCA3 expression was observed in urine samples of PCa patients compared to the benign group (Be-LGSc-> P=0.003, Be-HGSc-> P=0.014, Be-Ca-> P=0.001), while there was no discriminatory power between cancer subgroups (LGSc and HGSc).

The voltage-dependent anion-selective channel protein 1 (VDAC1) is a mitochondrial protein involved in the regulation of the metabolic and energetic functions of mitochondria and in mitochondria-mediated apoptosis. As a metabolite transporter VDAC1 is essential for energy production and cell growth and highly expressed in many cancers (126). Induced by hypoxia-generated factors VDAC1 promotes cancer cells to survive and was also implicated in chemotherapy resistance in lung cancer patients (182). Consistent with its tumor promoting function VDAC1 showed a significant difference between cancer subgroups (P=0.006, Bonferroni corrected) with higher expression in HGSc tumor samples of PCa patients.

Interestingly, it also showed almost significantly decreased expression in LGSc tumor compared with benign samples (P=0.075). This may indicate different roles of VDAC1 in tumorigenesis and progression.

α-Methylacyl coenzyme A racemase (AMACR), also known as P504S, plays an important role in fat metabolism and has high tissue specificity for prostate adenocarcinoma (59). In our exosome study AMACR is one of the “almost significant” genes (0.05<P<0.09), showing higher expression in HGSc cancer compared to benign samples (P=0.068). Increased damage of tissue and increased release of PCa-derived exosomes in HGSc cancers is a possible explanation for this pattern. This is also consistent with microarray studies that identified AMACR as an overexpressed tumor tissue marker for PCa (183-186). AMACR has also been detected in the blood of PCa patients to distinguish cancer from healthy patients (187). Recent studies demonstrate that AMACR IHC is positive in almost 100% of small PCa foci in needle biopsies but less intense in some uncommon prostate cancer types including atropic and pseudohyperplastic cancers. Moreover, androgen deprivation therapy as well as tumor de-differentiation as seen in high-grade cancer reduces AMACR staining in PCa (188).

Gene rearrangements, i.e. gene fusions and translocations have been implicated in carcinogenesis, initially especially in hematologic malignancies. These fusions can cause activation or inhibition of the transcripts of the fused genes. Recent genetic studies identified gene rearrangements and fusion genes as a common feature of prostate cancer (189, 190). One of these rearrangements implicates the fusion of the ETS transcription-factor gene ERG [(v-ets erythroblastosis virus E26 oncogene homolog (avian)] or ETV5 or ETV1 (ets transcription factors 5 and 1) with the gene encoding the androgen-regulated membrane-anchored serine protease TMPRSS2 (transmembrane protease, serine 2). Such a gene rearrangement is present in around half of all prostate tumors (191). It results in an androgen-driven, strong upregulation of the fusion gene transcript and subsequently of the ERG protein.

Detection of a fusion gene transcript by quantitative PCR in post-DRE urine may be of great help for PCa diagnosis, as this transcript is not present in benign lesions of the prostate. This rearrangement was reported to have 46% prevalence of PCa in prostate needle biopsies, and high level ERG and ETV1 gene expression has been found in patients with metastasis (192). In our study we have investigated the most common fusion transcript from TMPRSS2 exon 1 to ERG exon 4, which is expressed in approximately 85% to 95% of all fusion-positive PCa (193, 194). Only 6 of the 27 (22%) ERG gene rearrangement positive cases (by ERG immunohistochemistry in tumor tissue) yielded a positive urinary exosomes TMPRSS2-ERG

PCR result, while 16 samples positive by immunohistochemistry (IHC) had no detectable transcript in their exosome samples. The reason for this discrepancy could be; ***i.*** the tumor foci harboring the fusion gene has is well integrated into the tissue and a low number of exosomes are released into the urine (195), ***ii.*** the fusion gene harboring part of the tumor if far away from the urether and no exosomes enter the urine, and ***iii.*** the limitation of the assay detecting only 1 (although the most frequent) isoform of fusion transcripts. Thus, additional assays in urinary exosomes will be needed to detect other alternative isoforms. Hessels *et al*. analyzed urine sediments of 78 men with PCa-positive and 30 men with PCa-negative biopsies. They tested all transcript isoforms of the TMPRSS2:ERG fusion and established a PCR assay for urinary sediment samples with a sensitivity of 37% and they combined this assay with PCA3 detection to further improve the assay sensitivity (196). Another urine study by Laxman *et al* reported the detection of a fusion gene transcript (the most common isoform, TMPRSS2:ERGa) in urine sediment samples in 8 of 19 patients (64).

Considering TMPRSS2 and ERG single gene expression in the present study, TMPRSS2 alone was statistically significantly higher expressed in LGSc tumor compared to HGSc, (P=0.04), while ERG was highest expressed in HGSc tumors compared to the other groups and was found significantly different between benign and HGSc cancer (P=0.026). TMPRSS2 is an androgen-regulated gene like PSA. Considering that induction of TMPRSS2 mRNAs is dependent upon androgen receptor (AR) (197), our result suggests that AR transcriptional activity may have changed and dysregulated from LGSc to HGSc tumor progression.

Interestingly, a TMPRSS2:ERG fusion transcript has been detected in 2 “benign” samples in our study, in patients who had a negative biopsy 5 years ago. One of them was recently diagnosed positive for adenocarcinoma. Considering the positive detection of the fusion gene in the urines collected 5 years ago, urine exosomes may provide a novel sample type for detection of this fusion gene for early detection of PCa. It can show that urinary TMPRSS2:ERG fusion transcript detection may predict prostate cancer earlier than pathological detection, indicating the potential to use urine exosome RNA as a complement novel approach for PCa screening. However, it has to be considered that, although a TMPRSS2-fusion gene is highly specific for prostate cancer, a fusion gene is present only in approximately 50% of the cancers. Therefore, this assay has a high positive predictive value, but a much lower negative predictive value. Several efforts tried to overcome this problem by combining detection of a TMPRSS2-ERG fusion transcript with the level of the PCA3 transcript to better predict, whether urine contains PCa cells. Quantification of TMPRSS2:ERG and PCA3 in urine sediments after DRE improved the performance and enhanced the usability of serum PSA to predict PCa on biopsies.

In one study a detection sensitivity of 73% for the gene combination was reported, while the sensitivity for the fusion gene alone was only 37% and for the PCA3-based assay it was 62% (196). This combination to improve the PCa detection sensitivity should be further investigated in the future in urine exosome samples.

## 4.1 Conclusion and outlook

In this study we provide a proof of principle for a multiplex real-time PCR marker analysis in urinary exosome samples obtained from prostate cancer patients after digital rectal examination. A protocol was established for isolation, purification, amplification and PCR determination and for subsequent normalization and statistical evaluation. The 30 potential mRNA markers analyzed provide a promising starting point for the identification and testing of urinary exosome markers for prostate cancer. We identified gene markers that can distinguish Be vs Ca or HGSc vs LGSc. Particularly, PA2G4, ACTB and MME were found to be increased in benign urine samples, PCA3, PSA, KLK2 and PA2G4 were the best marker genes to separate cancer from benign patients, whereas TFF3 and VDAC1 were the most useful markers to distinguish low from high grade tumor patients. In future studies with more patients included, it will be necessary to confirm the usability of these mRNA markers. Our pilot study indicates that exosomes may provide an informative and useful resource for clinical PCa diagnostics. The further optimization of this method and test of potential marker candidates may help to improve PCa detection and to reduce unnecessary biopsies. On one side, tissue-specific exosome membrane proteins should be identified and used for enrichment of tissue-specific exosomes in order to improve the tissue specificity of this method. On the other side, the method for urine and exosome sampling, processing and biomarker detection could be further optimized to improve RNA yield and integrity and thus the detection sensitivity.

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# Abbreviations

| | |
|---|---|
| ABI | Applied Biosystems |
| ADT | androgen deprivation therapy |
| ANOVA | analysis of variance |
| AR | androgen receptor |
| Be | benign |
| BPH | benign prostatic hyperplasia |
| Ca | cancer |
| CV | coefficient of variation |
| DRE | digital rectal examination |
| EGF | epidermal growth factor |
| EtOH | ethanol |
| FDA | Food and Drug Administration |
| h | hour |
| HGSc | high Gleason Score |
| HKG | housekeeping gene |
| kDa | kilo dalton |
| LGSc | low Gleason Score |
| min | minute |
| MVB | microvesicular body |
| PCa | prostate cancer |
| PCR | polymerase chain reaction |
| PIN | prostatic intraepithelial neoplasia |
| PSA | prostate specific antigen |
| PSMA | prostate specific membrane antigen |
| RPE | radical prostatectomy |
| RT | reverse transcription (reverse transcriptase) |
| rpm | rounds per minute |
| TNM | tumor nodes metastasis |
| WTA | whole transcriptome amplification |

# Curriculum vitae

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