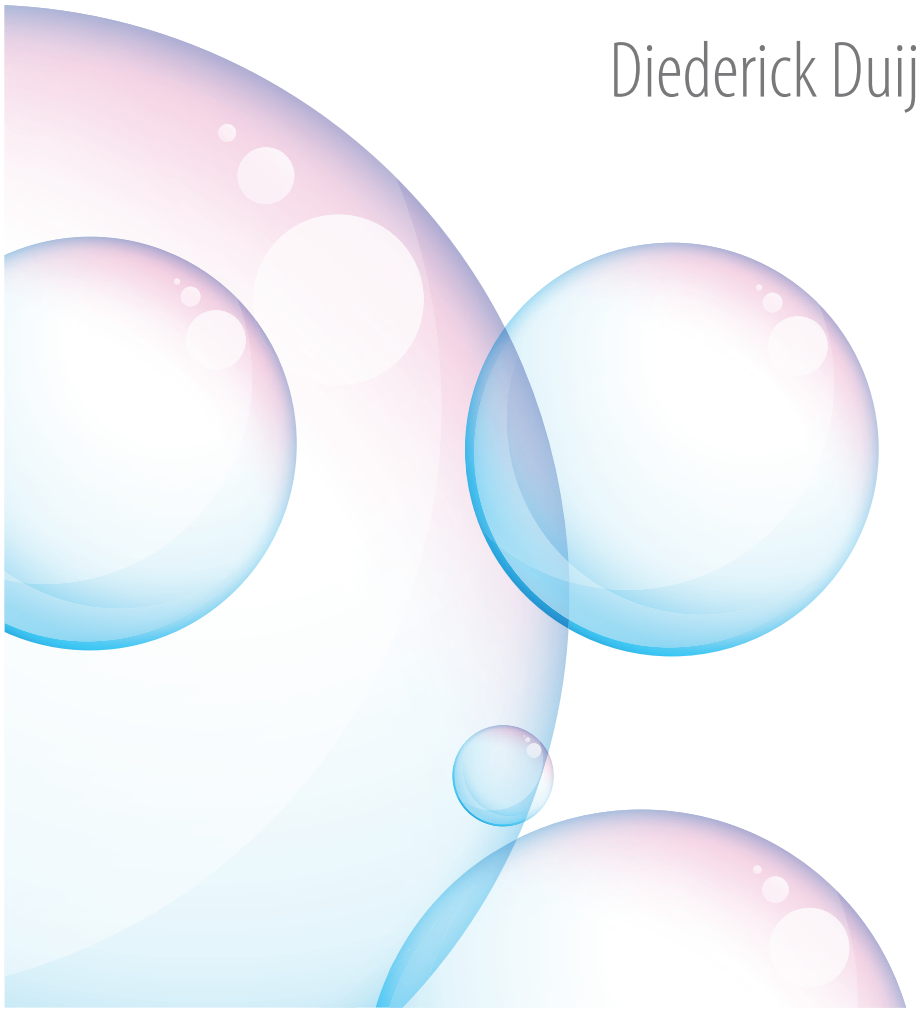
Extracellular vesicles as biomarkers for prostate cancer

Diederick Duijvesz



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Extracellular Vesicles as Biomarkers for Prostate Cancer

Extracellular vesicles als biomarkers voor prostaatkanker

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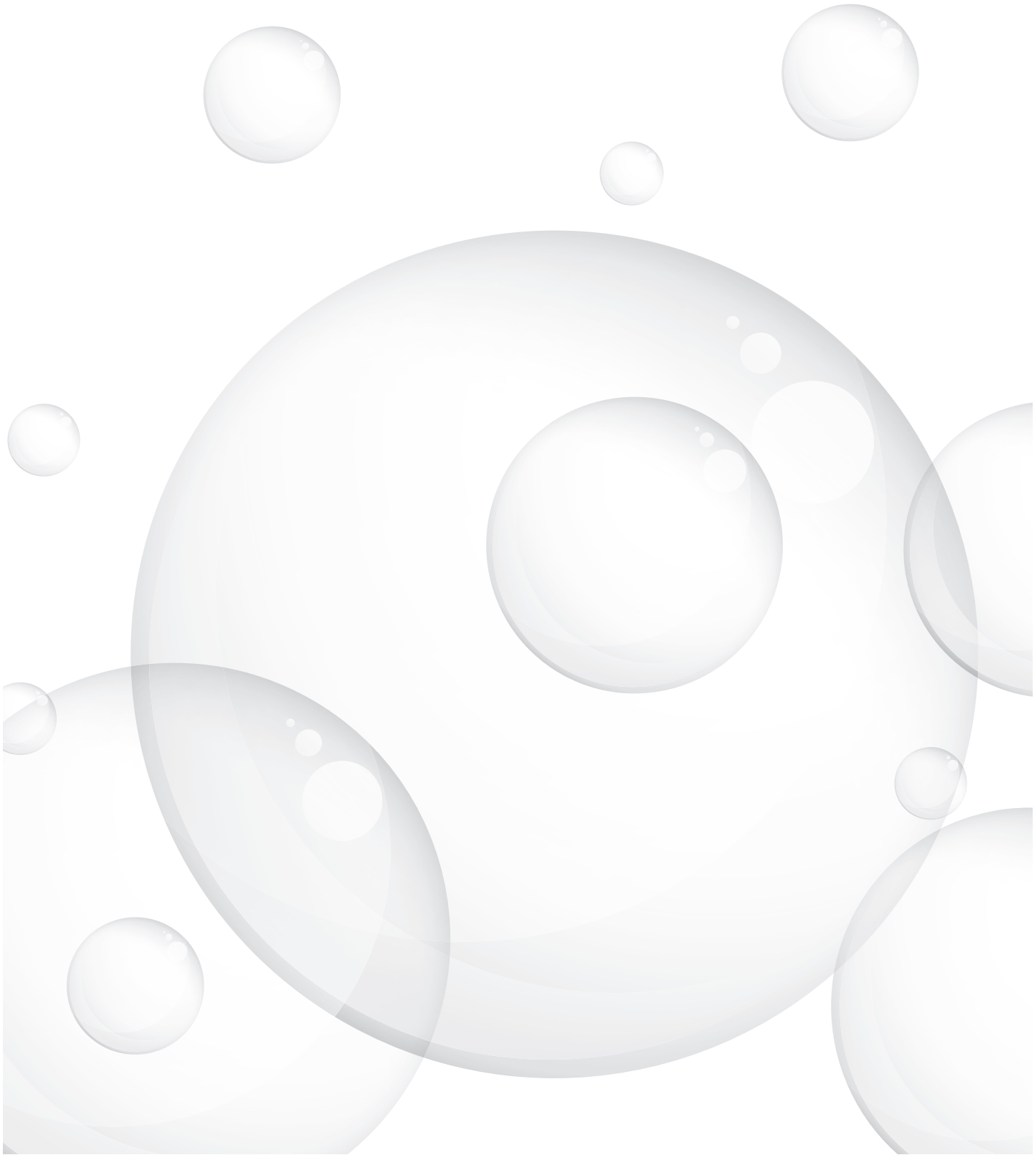
CONTENT



1 General introduction and outline thesis

THE PROSTATE

The prostate is a secretory gland that is part of the male reproductive system. It is located underneath the bladder and compromises the proximal part of the urethra. Ventrally it is attached to the pelvic bone with various ligaments. Dorsally it has a close relation with the rectum, which enables medical doctors to exam the prostate digitally via the rectum.

The main function of the prostate is production of fluid, contributing to approx. 30% volume of semen. This prostatic fluid contributes to the alkalinity of semen to neutralize the acidity of the vaginal tract, prolonging the lifespan of spermatozoa.[1] Epithelial cells also produce proteolytic enzymes such as prostate-specific antigen (PSA), which contributes in maintaining liquidity and mobility of spermatozoa after ejaculation.

Normally, the prostate has a size between 15-25 cc but with age the prostate can grow benignly.[2] Four different zones within the prostate can be identified.[3] The peripheral zone can take up to 70% volume (in young men), the central zone 25%, the transition zone 5% and the anterior fibro-muscular zone also approx. 5%. Most of the prostate cancers (70-80%) occur in the peripheral zone.

PROSTATE CANCER

In the Netherlands, the yearly incidence of prostate cancer (PCa) is approx. 11,000[4] and therefore, the second most common cancer among men after non-melanoma skin cancer. Each year 2800 men die because of PCa. In time, tumor cells can develop in the ageing prostate caused by accumulation of mutations in their DNA. These malignant cells have the potential to spread and form tumors throughout the body (metastases) and eventually lead to incurable disease.

In order to diagnose PCa early and prevent progression of the cancer, biomarkers are needed. Luckily, PCa is one of the few solid tumors with a clinically useful biomarker for both diagnostics and follow-up after treatment. This protein, PSA, has been considered the "gold standard" for the detection of PCa.[5] Although PSA has acceptable sensitivity, it lacks specificity. Furthermore, PSA-based screening leads to a high risk of overdiagnosis and overtreatment based on findings on complementary diagnostic prostate biopsies.[6,7] Therefore, new molecular markers for PCa are needed.

TUMOR MARKERS

A tumor marker in a biomedical setting can be defined as 'a biological object present in human tissue and/or body fluids that is capable to differentiate between normal and

abnormal biological conditions'.[8] The National Institute of Health added that it should be measured objectively and is evaluated as an indicator of pathogenic processes or biological responses to a therapeutic intervention. With this definition a wide range of characteristics can be used as a tumor marker, such as easily observable skin lesions, MRI-scans, or more inconspicuous variables such as proteins or RNA present in tissue, serum or urine. Nowadays, the term tumor marker is inextricably linked to molecular markers.

So far, different kinds of tumor markers have proven to be a useful diagnostic or prognostic tool for medical doctors when assessing a certain disease, especially within the field of oncology. The presence or an elevation of a marker could indicate the existence of a malignant tumor. Furthermore, it could also have the ability to predict disease development or outcome upon treatment. Also in PCa, tumor markers have been widely used in daily clinical practice. This chapter will discuss multiple types of tumor markers for the diagnosis and prognosis of prostate cancer and will review a selection of markers that have been validated to some extend or are of high interest.

DIFFERENT TYPES OF MARKERS

Tumor markers can be classified into several categories with their own specific purpose. The different kinds of markers can describe the chance of getting a disease (risk marker), the presence of disease (diagnostic marker, early detection or screening marker), how the course of the disease will be (prognostic marker), to estimate the chance of success of a certain treatment (predictive marker).[9] Furthermore, markers can also be applied to observe therapy efficacy during or after treatment (monitoring marker).

- When using a marker for risk assessment, the disease is not yet (clinically) present or cannot be detected with conventional techniques. Such a marker would be mainly suitable for life-threatening diseases that are typically diagnosed too late. In addition, risk markers can be implemented to identify a subpopulation for regular checkup or screening. In recent years, much research has been dedicated to the identification of genomic changes using genome-wide association studies (GWAS) to identify single nucleotide polymorphisms (SNPs) associated with the development of a disease.[10] For PCa, it is evident that many of such SNPs are linked to disease development, although none of them individually have a very strong correlation.[11]
- Diagnostic markers have the ability to determine the presence or type of malignancy. Such a marker is often used in immunohistochemically examination of tissue specimens or in specific protein/mRNA analysis of patient-derived body fluids.
- Prognostic markers become very useful when it is possible to stratify patients in groups that have different outcomes. Based on this stratification, the physician can

choose a specific therapeutic option in order to individualize treatment. Next to the choice of treatment, if aggressive subtypes can be identified, treatment can be initiated earlier.[12] One of the best prognostic markers for prostate cancer is Gleason score, a representation of the organization of tumor glandular architecture.[13]

- Predictive markers are used to foretell the responsiveness to or outcome of a specific treatment. Although some markers have been described that predict the efficacy of hormone, radiation or chemotherapy, these markers are not yet utilized in clinical practice.
- Monitoring markers are measured before, during and after treatment to determine effectiveness of therapy. Prostate specific antigen (PSA) is a highly effective and established monitoring marker for efficacy of radical prostatectomy, hormone therapy and/or radiotherapy.[14]

The occurrence, elevation or modification of tumor markers can be caused by several biological processes (Table 1). Some endogenous cellular products are produced and shed at a greater rate by the abnormal cancerous cells. Also, these markers can be released differently due to a higher apoptosis and necrosis rate in cancer. Furthermore, markers can reveal themselves when the environment of the cells becomes aberrant. An example is PSA, where higher levels in serum are be detected when the blood-prostate barrier is affected. In addition, products of newly created genes in cancerous cells, such as the TMPRSS2:ERG fusion transcript, are applicable as highly specific markers. Regarding prostate cancer, DNA (genomics), mRNA (transcriptomics), proteins (proteomics) and metabolites (metabolomics) have been the biochemical analytes investigated that could contribute to a better and more precise diagnosis and prognosis.

Table 1. Expression of different kind of markers in healthy tissue as compared to malignant tissue

Healthy tissue	Malignant tissue	Type of dysregulation	Example marker
+	+++	Upregulated in cancer	AMACR/PCA3
+	+	New distribution due to cancer	PSA
-	+	Mutation, Oncogene	TMPRSS2:ERG
+	-	Mutation, Tumor suppressor	PTEN
+++	+	Downregulated in cancer	GSTP1

BIOLOGICAL MATERIALS FOR TUMOR MARKER ANALYSIS

When searching for new tumor markers it is important to choose which biological material to explore. The most logical material is the one for which eventually a clinical applicable assay can be generated.[15] Therefore, samples derived with minimally-invasive techniques and those easily obtainable, such as blood or urine, are the most obvious.

Blood is widely used, mainly because of the traditional availability and of the idea that biochemical analytes in plasma might provide important insight in disease specific characteristics. Unfortunately, discovery of tissue or cancer specific marker is hampered by the abundances of all kinds of different analytes. The abundant proteins are identified preferentially and are generally not useful cancer markers. Probably the most interesting new tumor markers are present in the low abundance range. Unfortunately, for certain technologies such as mass spectrometry, the high abundant analytes overshadow the detection of the low abundant ones. This problem is in essence the so called 'dynamic range problem'. As an example, the proteome in blood has shown to consist of 3500 proteins so far, but many more have to be identified.[16] The 22 most abundant proteins account for 99% of the measured proteins, so the search for new and low abundant tumor markers is like searching for a 'needle in a haystack'.[17]

Another issue that arises when using materials such as blood is the origin of the marker. Like most clinically applied cancer markers, it is expected that the disease-specific markers are derived from the cancer cells or organ of origin. When candidate tumor markers are identified in serum, it is difficult to determine from which tissue these markers originate. It becomes slightly less complicated with the use of urine or prostatic fluids/seminal fluids. These materials are more specifically related to the prostate and the abundance and variety of analytes is generally much less.

IDENTIFICATION AND VALIDATION OF NEW MARKERS

Discovery phase

Discovery of new markers is often an open and unselective search by which the differential expression of specific biochemical analytes between states is first defined.[15] If one wants to identify a specific marker, optionally, two separate states have to be compared without the influence of confounding factors (Figure 1). This comparison and eventual identification are typically performed with state-of-the-art technologies such as mass spectrometry or microarray analysis by using a small training set of samples. Drawbacks from this phase are the costs and the limited number of samples that can be analyzed. Because of the limited number of samples and the large number of analytes tested, many top candidate markers will be false positives and some genuine markers will not be significantly different (false negative).[15] With statistical calculations for false discovery rate and multiple testing corrections, these false positively identified analytes can be trimmed down. Eventually, after a list of potential tumor markers is generated, a more focused approach has to be taken where the most promising candidate markers must be validated.

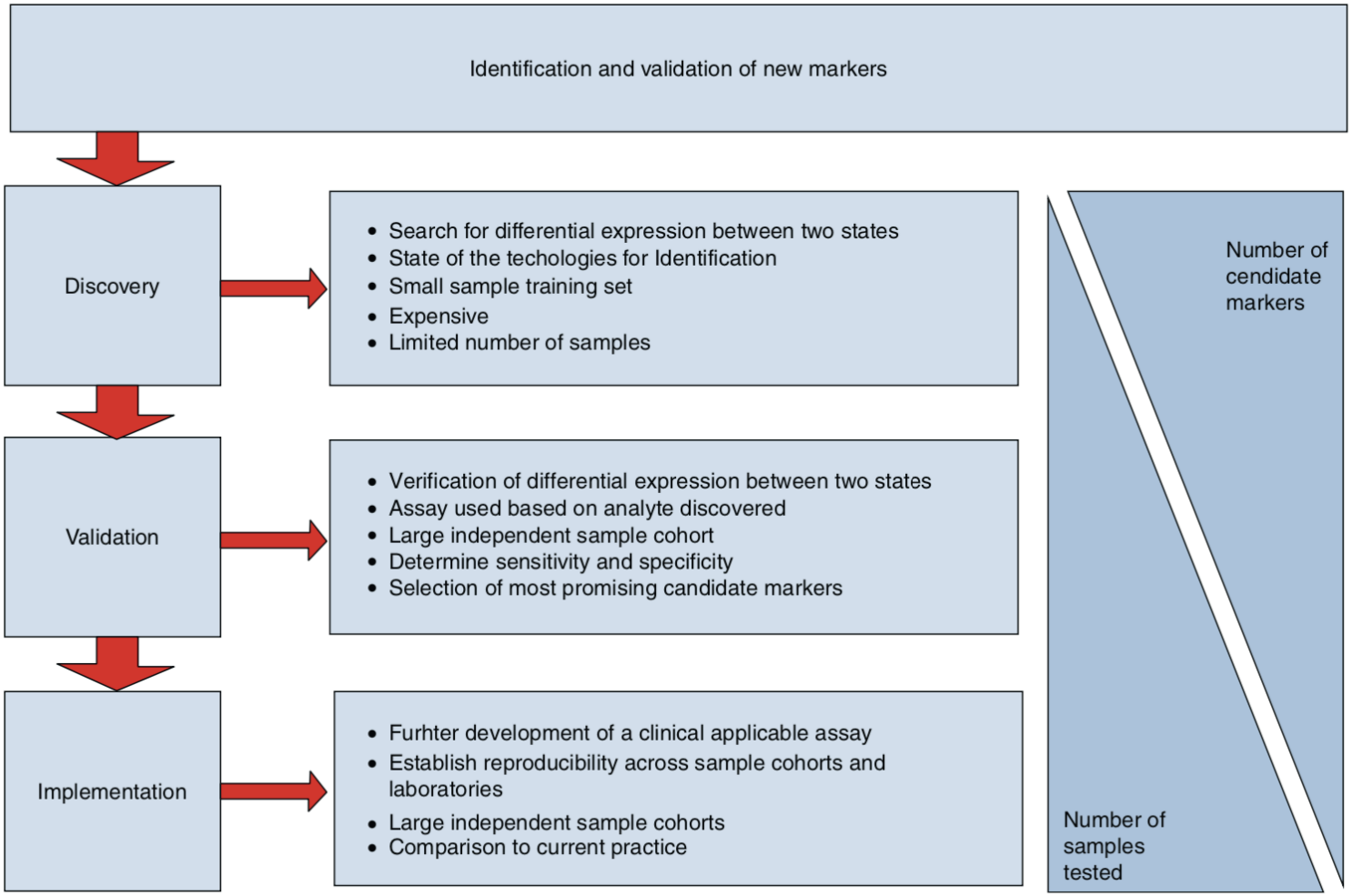


Figure 1. Identification and validation of new markers

Validation phase

The validation phase verifies the differential expression between samples and will give the opportunity to test the candidate tumor marker in an independent cohort (validation set). For this phase an assay has to be developed that is capable of accurately measuring the candidate markers. The assay that is preferentially used is based on the specific analyte that has been discovered. For example, if a specific protein is identified, an ELISA (enzyme-linked immunosorbent assay) is typically a very sensitive and reliable test. When RNA is the marker of interest, most likely the assay that will be used is RT-qPCR (reverse transcriptase quantitative polymerase chain reaction). Besides these already established and widely used tests, novel techniques can be developed in order to more easily or more accurately detect the new tumor markers.[18,19] Finally, with a specific and reliable test available, it has to be administered to larger study cohorts in order to test the most promising candidate markers. This cohort has to contain specific variables in order to evaluate its restrictions and indicate the exact disease characteristics for which this candidate marker is most suitable. These experiments aim at confirming the previously discovered markers and will show their sensitivity and specificity for the particular disease it has been identified for. Eventually, from this validation step, only a few promising candidate tumor markers submerge. The ones that show a positive correlation with disease specific characteristics will be used for the development of a clinical applicable assay. Normally, the whole process extends over a time line of at least 5 years, where initially 100-1000 analytes are identified in the discovery phase. Unfortunately, only very few, if any, will survive the validation phase and reach the clinical implementation phase.

Implementation phase

In this phase the main focus is the further development of a clinical applicable assay that can be used to further validate and implement the tumor marker. With the assay development it is important to establish reproducibility across independent cohorts and laboratories.[20] By using this test, its operating characteristics are evaluated and a certain clinical cut-off value further tested and adjusted in multi-center prospective studies and compared to current practice. Only after this last phase a specific test will gain wide acceptance and eventually be applied in a clinical setting.

OBJECTIVE OF THIS THESIS

Since current molecular biomarkers lack specificity or sensitivity for PCa diagnostics, new and better markers need to be identified. The main objective of this thesis is the identification of novel candidate biomarkers for PCa by profiling extracellular vesicles.

Chapter 2 provides an overview of known and (clinically) used PCa markers. It describes the clinical use of PSA, its isoforms and a range of other markers. Because the search for new and better biomarkers is hampered by the dynamic range problem, several techniques can be applied for selection and enrichment. One of those techniques is the isolation of extracellular vesicles. These vesicles contain a selection of proteins and/or RNAs that reflect cellular conditions from the cell they were shed. **Chapter 3** introduces extracellular vesicles and explains it potential as a biomarker 'treasure chest'. It also gives an update on the work that has already been performed regarding these vesicles within the field of Urology when this thesis was initiated.

In **chapter 4** we address the discovery phase of biomarker detection by proteome profiling of extracellular vesicles. In collaboration with the Environmental Molecular Science Laboratory (EMSL), Richland, WA, USA, we aimed to identify proteins from vesicles released by prostate cancer cells and immortal normal prostate cells. Using mass spectrometry and various techniques to verify our findings, we identified a series of proteins that were more abundant in vesicles from cancer cells as compared to normal prostate epithelial cells.

Our second objective was the validation of novel candidate biomarkers for prostate cancer on patient tissue samples. In **chapter 5** and **chapter 6** we describe the use of tissue mass spectrometry and an extensive tissue microarray to validate a few markers of interest. With these techniques we explored the diagnostic and prognostic potential of selected candidate biomarkers for PCa.

Unfortunately, current techniques for isolation and characterization of extracellular vesicles are labor intensive and unsuitable for daily clinical practice. Therefore, our third objective compromises the development of a clinically usable (high-throughput) assay to analyze extracellular vesicles from patient samples (urine or serum). In **chapter 7** we describe the results of our collaboration with the department of Biotechnology of the University of Turku, Finland. Together we developed a fast, highly sensitive and reliable immunoassay (TR-FIA) that can be used for clinical implementation.

Finally, in part 3 all findings are summarized, a general discussion is provided and future perspectives recited.

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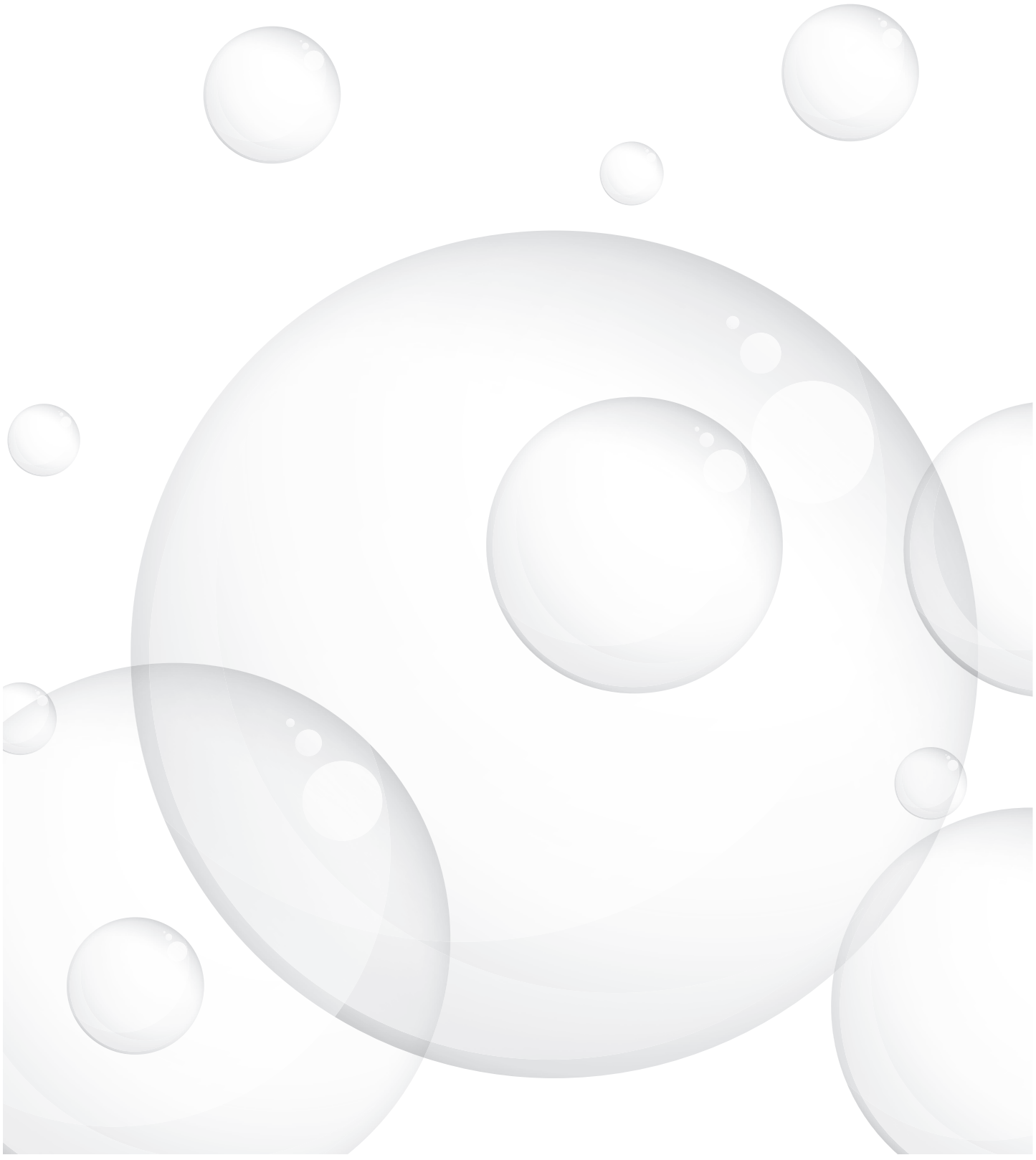

2

Tumor markers in prostate cancer

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Novel tumor markers for prostate cancer are still needed to improve the ability to detect prostate cancer, predict prostate cancer related morbidity and mortality and monitor response to treatment. Current markers used in research and even in the clinic remain controversial (Table 1).[1] The most widely applied biomarker in prostate cancer is PSA. Because of its limitations, multiple new markers have been evaluated to compensate for these limitations. Unfortunately many of these markers have not made it into the clinic, which shows that identification of better markers remains a challenge.[2]

Table 1. Current tumor markers for prostate cancer

Marker		Biological function	Biochemical analyte	Marker ability
PSA	Prostate specific antigen	Serine protease	Protein	Screening/ Diagnosis/Prognosis
%fPSA	Percentage free PSA		Protein	Diagnosis/prognosis
PSAD	PSA Density		Protein	Diagnosis/prognosis
PSAV	PSA Velocity		Protein	Diagnosis/prognosis
[-7],[-5],[-4],[2] ProPSA	PSA isoforms		Protein	Diagnosis
hK2/KLK2	Human Kallikrein 2	Peptidase, cleaving proPSA to mature PSA	Protein	Diagnosis
PCA3	Prostate cancer antigen	Non-coding mRNA without a functional protein	RNA	Diagnosis
ETS	E twenty six gene family	Chromosomal rearrangement without a function	DNA	Prognosis
TMPRSS2:ERG	Trans membrane protein serine 2 (TMPRSS2) and ETS related gene (ERG)		DNA Protein (ERG)	Prognosis
AMACR	Alpha-methylacyl coenzyme A racemase	Metabolization of fatty acids and bile acid biosynthesis	RNA Protein	Diagnosis/prognosis
GSTP1	Glutathione S-transferase pi 1 (methylated)	Detoxification of carcinogens	DNA	Diagnosis/prognosis
PSMA (FOLH1)	Prostate specific membrane antigen	Peptidase, hydrolyzing peptides in prostatic fluids	RNA Protein	Prognosis
PSCA	Prostate stem cell antigen	Membrane based glycoprotein	RNA Protein	Diagnosis/prognosis
CgA	Chromogranin A	Proteolytic protein	Protein	Prognosis
B7-H3	Transmembrane protein family B7, member H3	Regulation of T-lymphocytes	Protein	Prognosis
CAV1	Caveolin-1	Molecular transport, cell adhesion and signal transduction	Protein	Diagnosis/prognosis
GOLPH2	Golgi phosphoprotein 2	Sorting and modification of proteins through the Golgi apparatus	RNA Protein	Diagnosis

Table 1. Current tumor markers for prostate cancer (continued)

Marker		Biological function	Biochemical analyte	Marker ability
CRISP3	Cysteine-rich secretory protein 3	Unknown	RNA Protein	Diagnosis/Prognosis
Sarcosine		Metabolite produced after enzymatic transfer of a methyl group from S-adenosylmethionine to glycine	Protein (metabolite)	Prognosis
Exosomes	Nano-sized vesicles, 100 nm in diameter containing RNAs and proteins	Intercellular communication, part of degradation pathway	RNAs and Proteins	Diagnosis/prognosis

PSA

Since its discovery in 1970, PSA has revolutionized the diagnosis and management of prostate cancer.[1] Subsequently, after its application in urological practice it has proven to be a valuable tool for (early) detection, staging and monitoring of men diagnosed with prostate cancer (Figure 1A).[3,4] Especially the use of PSA as a screening tool has increased the identification of prostate cancers and also improved curability with treatment.

PSA, also known as KLK3 or hK3, is a member of the human Kallikrein family. This gene family consists of 15 members and is described with a distinct nomenclature.[5] The first three members (hK1, hK2 and hK3) encode for serine proteases that have diverse physiological functions. Expression of PSA and some other Kallikrein members is androgen regulated. PSA protein has a half-life of 2-3 days and is secreted by prostatic epithelial cells into seminal fluid. Most likely through tissue leakage, PSA can be found in serum, but with a concentration of about 10^6 times less as compared to seminal fluid.

Initially, PSA is produced as a 261 amino acids preproenzym with a 17 amino acid signal peptide that is removed during synthesis (Figure 1B).[6] After this step, proPSA is formed which contains 244 amino acids, from which subsequently 7 amino acids are cleaved so it is processed to PSA that contains 237 amino acids. When shed in serum, PSA is unbound (free PSA or fPSA, 5-35%) or bound (complexed PSA or cPSA) to complexes with the antiproteases α(alpha)1-antichemotrypsin (PSA-ACT), α(alpha)2-macroglobuline (PSA-A2M) or α(alpha)1-protease inhibitor (PSA-API) which inactivate its function.[7] In seminal fluids it functions as a protease that liquefies semen by interacting with semenogelin and firbronectin.[8,9] Although PSA is highly specific for prostate epithelial cells, in much smaller concentration it can be measured in malignant breast cells, salivary gland, bowel, other urological tissues and renal carcinoma cells.[10-12] Nevertheless for practical and clinical purposes PSA is organ specific because after removal of all prostate tissue PSA values become immeasurable in serum. Although PSA is organ specific, it cannot be ascribed

as prostate cancer specific because other urological conditions such as benign prostate hyperplasia (BPH), prostatitis or mechanical damage also contribute to aberrant PSA-values in serum.[13] It is noteworthy that the production of PSA by prostate cancer cells is not higher than benign prostate epithelial cells, but higher serum values is a result of an altered prostate-blood barrier.[14] In fact, production of PSA by prostate cancer cells is generally lower.[15]

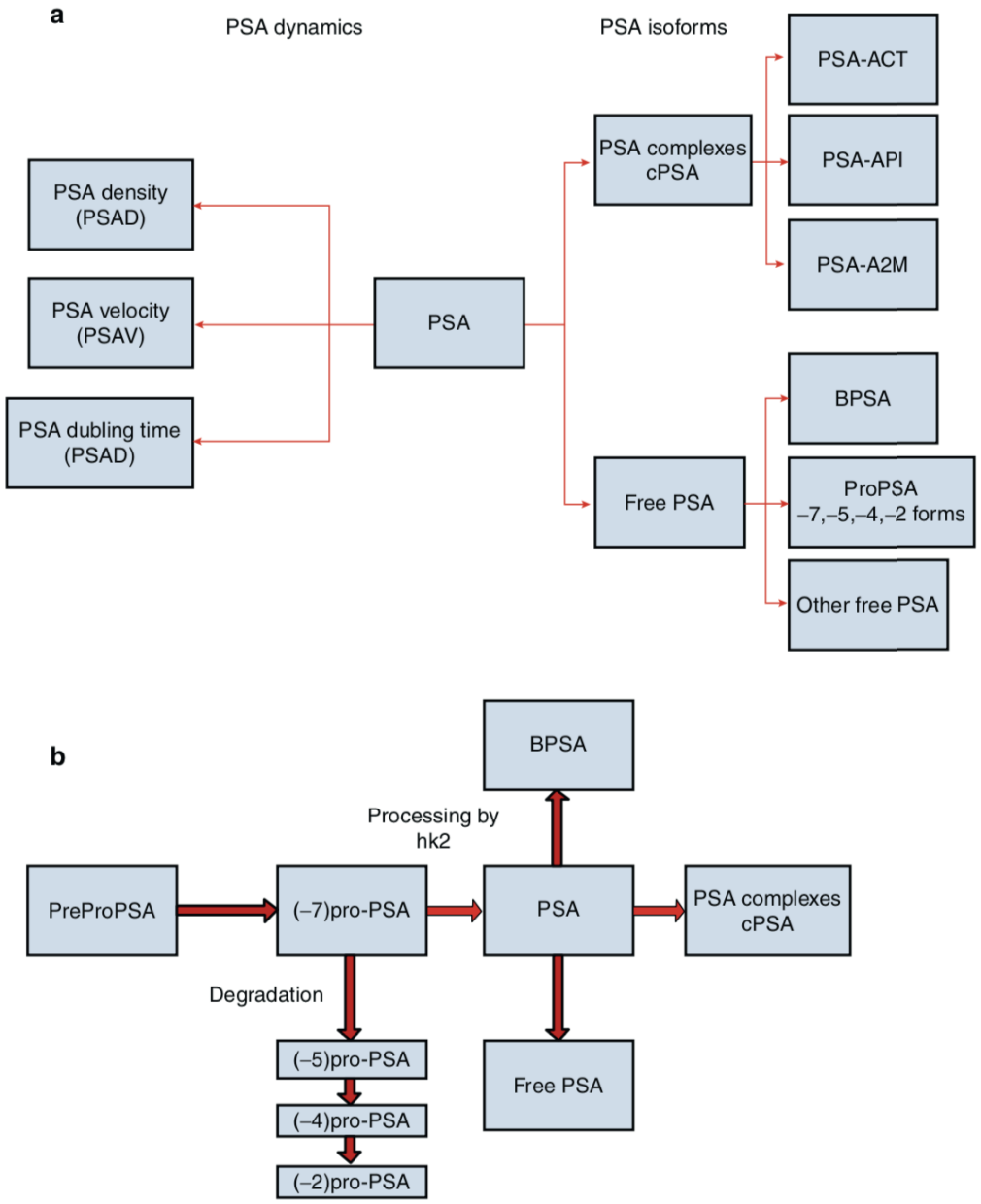

Figure 1. A. Different measurements contributing PSA including PSA dynamics. B. Processing of PSA to its subforms.

Large studies showed that 97% of all men older than 40 years have PSA serum levels lower than 4 ng/mL, which gave rise to the idea that this value should be the threshold when it is used in a diagnostic setting.[16] Furthermore, it was shown that PSA serum values could increase when prostate cancer is present.[17,18] Initially PSA was used as a reliable marker to prove residual disease or progression after radical prostatectomy for prostate cancer.[19] Patients with lower values preoperative had higher rates of organ-confined disease.[20,21]

In a screening setting it has been shown that PSA can increase the detection rate of prostate cancer in men without symptoms.[22] By using PSA, the percentage of men who were found with metastases at diagnosis was reduced from 16% to 4%, but also late-stage disease and prostate cancer related mortality was observed to be less.[23] During the last decades it is shown that with the use of PSA the detection of prostate cancer has increased dramatically, but that prostate cancer mortality was only reduced with 20%. Therefore it was concluded that using PSA for the detection of prostate cancer results in a substantial overdiagnosis and overtreatment.[24]

As a diagnostic tool PSA has a high sensitivity but low specificity for prostate cancer, where the positive predictive value (>4.0 ng/mL) is limited to 25%.[25,26] Serum PSA levels are influenced by tumor grade, volume and site of origin (primary tumor or metastases) and it is capable to predict pathological features.[13] On the other hand, in 15% of men with low PSA levels, prostate cancer is present.[27] So, in order to improve identification of prostate cancer and gain specificity, changes in variant forms of PSA have been investigated and introduced into the clinic.

FREE PSA

The proportion of free PSA (%fPSA) is lower when compared to total PSA in healthy men or men with BPH.[28-30] Therefore, %fPSA has been suggested as a marker for prostate cancer.[31] The exact cause for this occurrence is not fully understood, but it is thought that in patients with prostate cancer PSA 'escapes' proteolytic activity and stays bound to ACT, A2M or API. An extensive meta-analysis that compromised 66 studies showed that %fPSA and cPSA have better diagnostic potential compared total PSA (tPSA) in the intermediate range of 2-10 ng/mL.[32] In studies where %fPSA is combined with serum PSA levels between 2.5 and 4 ng/mL, more specificity can be obtained in diagnosing prostate cancer.[33] The use of %fPSA could contribute to a more reliable diagnosis and therefore maybe reduce biopsies by 20% and lessen the overdiagnosis.[34] Furthermore, a better stratification could be made of patients who are more eligible to undergo active surveillance and therefore decrease overtreatment.

As a prognostic marker, high %fPSA correlated with smaller and lower grade prostate cancer.[34] Vice versa low %fPSA resulted in a more aggressive form of prostate cancer, even when measured up to 10 years before diagnosis.[35] Prostate cancers with Gleason scores of >7 and extra capsular extension also showed a correlation with low %fPSA.[36,37]

PSA DENSITY

In a majority of men with slightly elevated PSA levels, the main contributor is probably BPH and only in a small percentage of men, prostate cancer.[36] To differentiate better between these two condition a method was introduced that compensated for the increase of serum PSA levels by prostate enlargement.[38] This measurement, PSA density (PSAD) where serum PSA is divided by prostate volume (>0.15), has shown to have a direct relationship with the probability of having prostate cancer, especially with intermediate PSA levels and no abnormalities on DRE (digital rectal exam).[39,40] Although these primary reports embrace promising results, this measurement has shortcomings. When PSAD was compared to PSA it was not able to enhance the predictive value of PSA alone.[41] Furthermore, PSAD in not sensitive enough for prostate cancer detection, almost 50% of all cancers are missed.[42] The most plausible interpretation of these conflicting results is most likely the heterogeneity of prostate volumes in prostate cancer and BPH. Because PSAD is influenced by prostate volume, the number of epithelial cells has to be a correction for these factors. Correction for transition zone size has shown to be a very specific and sensitive technique to detect prostate cancer, but because of the variability of ultrasound measurements it has not gained wide acceptance in daily practice.[43] Also as prognostic marker, increased PSAD values were correlated with Gleason scores >7 and a greater risk of organ confined disease.[44]

PSA VELOCITY

Another approach for detecting prostate cancer in the intermediate range of serum PSA is by using PSA velocity (PSAV), where the rate of PSA change between two separate measurements is taken into account.[45] As a diagnostic tool, an increase of 0.75 ng/mL or more per year is correlated with the presence of prostate cancer, which has a high specificity with PSA values between 4-10 ng/mL (up to 90%).[45,46] To obtain a reliable PSAV result, the interval between the two separate measurement should be at least 18 months.[46] This interval seems not to be optimal for clinical daily practice because it can cause a delay in treatment. Furthermore, based on the characteristics of this marker, its use is limited. When initial PSA values are less than 4 ng/mL the sensitivity and specificity

is dramatically reduced.[47] As a prognostic marker, increased PSAV is significantly related to aggressiveness. One study showed that preoperative PSAV values of >2.0 ng/mL per year resulted in a nine times higher chance of prostate cancer related mortality after prostatectomy or external beam radiotherapy.[48,49] A recent study revealed that even a PSAV of >0.35 ng/mL per year correlated with a significant higher chance of biochemical progression.[50] On the other hand, when values of <0.4 ng/mL per year were used, it increased the likelihood of insignificant prostate cancer.[51] Besides these promising results, the exact role of PSAV in the stratification and characterization of specific subgroups of prostate cancer patients remains not fully elucidated. More research has to be performed to maximize its potential as a tumor marker and to establish the most ideal cutoff PSAV value for diagnosis and determining prognosis.

PSA DOUBLING TIME

Closely related to PSAV, PSA doubling time (PSADT) could also harbor some interesting capacities as a tumor marker. PSADT is defined as the time that serum PSA levels are doubled. As a diagnostic tool, so far no reports have been published. Nevertheless, the predictive abilities of this tumor marker has been the focus of multiple research efforts, but their results show no relationship between pretreatment PSADT and post treatment outcomes.[52] As a prognostic marker it has mainly been measured post prostatectomy and was correlated with survival results. The first study showed that fast PSADT values (<10 months) correlated with lower metastasis-free survival.[53] Others showed that if PSADT was <3 months within a period of 24 months after radical prostatectomy there was an associated with lower cancer specific survival.[54]

PSA ISOFORMS

ProPSA is an inactive precursor of PSA that is cleaved by hK2 or hK4, converting it into its active form.[55] The precursor form of PSA contains a 7 amino-acid proleader peptide and is therefore named [-7]proPSA. Incomplete cleavage of proPSA results in other subforms, such as [-2], [-4] or [-5]proPSA. Elevated levels of proPSA and its truncated forms were observed in prostate cancer tissue.[56,57] A possible explanation for this finding was the observation that proPSA is higher expressed in the peripheral zone of the prostate.[57]

Mainly in the intermediate range (2.5-10.0 ng/mL) of PSA, ProPSA could early detect more prostate cancers.[58-60] Even when these isoforms were used, it could avoid 59% of all biopsies taken, as compared to 33% when only %fPSA was used. Unfortunately, in a prognostic setting proPSA does not seem to be superior to %fPSA, but when combined

it is correlated with higher Gleason scores and non-organ defined prostate cancer.[61] All the single sub-isoforms of proPSA have been investigated and showed no better correlation in diagnosing or determining prognosis as compared to total proPSA or %fPSA.[60]

KLK2

Human Kallikrein 2 (hK2 or KLK2) is also a member of the Kallikrein family and shares 80% homology with PSA. It functions as a peptidase, cleaving proPSA to mature and active PSA.[62,63] Like PSA, it is highly and specifically expressed in the prostate and is androgen regulated. hK2 levels show a distinct expression pattern on immunohistochemical analysis, which was also observed in serum. These findings indicated that this marker could be indicative, independent of PSA.[64,65] The first studies on hK2 showed no correlation of this marker with prostate cancer.[66-68] Nevertheless, a review that also included all studies on hK2 performed in a later stage, revealed a significant higher expression of hK2 in serum from prostate cancer patients.[60] Especially for the intermediate elevated PSA values, it showed a better discrimination as compared to %fPSA. As a prognostic marker hK2 is capable of differentiating between low and high Gleason scores and also for extra-prostatic growth, even prior to radical prostatectomy.[69-71] Unfortunately, when this marker was analyzed in a multivariate model it had a very limited improvement on prognoses as compared to Gleason score alone.[72,73] One study revealed that hK2, together with other variables, was significantly predictor of biopsy outcome.[74]

URINARY PSA

In almost all reports, PSA as a tumor marker for prostate cancer was measured in serum. In contrast to serum PSA, also urinary levels of PSA were evaluated as a potential tumor marker for prostate cancer.[75] Although the first report was published in 1985, less is known about this PSA measurement. Just as serum PSA it was shown that elevated urinary PSA after radical prostatectomy was correlated with disease recurrence and therefore was suggested as a monitoring marker.[76] In a diagnostic setting, when a ratio was taken of urinary and serum PSA expression it was shown that it produced higher sensitivity and specificity as compared to serum PSA alone, especially in the intermediate range.[77,78] Unfortunately, reports on urinary PSA levels are few and more research is needed to fully elucidate if urinary PSA has any potential as a marker for prostate cancer.

PCA3

The PCA3 transcript (prostate cancer antigen 3) was discovered in the late 90s as a new promising candidate marker for prostate cancer.[79] The PCA3 gene is located on chromosome 9q21-22 producing a (non-coding) mRNA that does not encode a protein.[80,81] After its discovery it was named DD3 (differential display clone 3) as a result of a differential display analysis that was used to compare mRNA expression between healthy prostate tissue and prostate cancer tissue.[82] 95% of prostate cancer specimens highly expressed PCA3, compared to no expression in normal prostate, BPH or other types of cancerous tissues. High grade PIN also revealed higher expression, up to 96% of the cases.[83,84] PCR on similar samples showed a 66-fold increase in PCA3 expression in prostate cancer samples with a sensitivity of 94% and specificity of 98%.[85,86] Furthermore, the expression of this marker is not influenced by age, prostate volume and infections.[82] The current PCA3 test is mRNA based and the outcome is a ratio between PCA3 mRNA and PSA mRNA multiplied by a 1,000.[86] This test is preferentially performed on urine samples that are collected after digital rectal examination or prostate massage.[87] When this test is performed on serum, it has less accuracy.[88]

Initially, the PCA3 test was launched to predict presence of PCa after negative biopsies. Subsequent reports on the urine test showed a sensitivity of 54-82% with a specificity of 66-83%, where PSA has a sensitivity of only 22-47% for the diagnosis of prostate cancer.[82,84,86,88-90] Multiple studies have shown that increased PCA3 is statistically significantly correlated with more tumor volume.[91-93] PCA3 also outperformed the diagnostic accuracy of %fPSA. This diagnostic accuracy can even further be increased when PCA3 is combined with other (clinical) variables such as PSA, physical characteristics during digital rectal examination, age and family history.[94] In a screening setting, PCA3 was capable of improving the performance characteristics and identification of serious disease compared with PSA.[95]

Although many reports describe the relation and prognostic features, such as histopathological outcome, generally no correlation could be observed between PCA3 and Gleason score and pT staging.[96] With these data it was suggested that PCA3 could be applied to predict histopathological outcome after biopsy, especially in patients with elevated PSA and a negative biopsy.[90,96,97] Furthermore, it was suggested that PCA3 could be used to determine multifocality of prostate cancer lesions and patients that are candidates for active surveillance.[82,98-100] The exact role of PCA3 in determining diagnosis and prognosis of prostate cancer remains to further investigated. Since the PCA3 detection assay is RT-PCR (reverse transcriptase PCR) based, the assay needs to be performed by expert labs and is much more expensive than protein-based ELISAs.

ETS

In prostate cancer, chromosomal rearrangements affecting the ETS (E twenty six) gene family members are common events; around 60-70% of all cases exhibit such an alteration.[101,102] In a majority of the rearrangements there is a fusion between the genes TMPRSS2 and ERG, the so called TMPRSS2:ERG fusion gene, which is unique for prostate cancer. Both TMPRSS2 and ERG genes are located in the same orientation on the long arm of chromosome 21. They are spaced by approx. 3 million base pairs and a deletion of this interstitial region can cause fusion of the two genes. Because the TMPRSS2 gene is androgen regulated, a fusion of this gene with ERG results in the androgen regulated and high expression of ERG. So far, this fusion is never observed in normal tissue and unique to prostate cancer.[103]

Multiple gene fusion partners that are related with either the TMPRSS2 part or the ERG part have been identified.[104] Other fusions of the TMPRSS2 gene occur in fewer cases with ETV1, ETV4 and ETV5. Although the TMPRSS2 gene is most often involved, other fusion partner such as the SLC45A3, ACSL3, HERV-K, FOXP1, EST14, KLK2, CANT1, DDX5 genes can rearrange with ETS family members.[105] All these gene fusions are unique to prostate cancer and seem to play an important role in the biogenesis and development of this disease. Therefore they could function as marker for diagnosis and prognosis. Recent studies showed that the fusion of TMPRSS2 to ERG is present in the precursor lesions PIN (prostatic intraepithelial neoplasia) and therefore must be an early event in cancer development.[106,107] Multiple studies that address the prognostic value of this marker have been performed, with several opposing conclusions.[102,105] Two studies examined 114 and 150 prostates after radical prostatectomy and revealed that expression of ERG or TMPRSS2:ERG correlated with a reduction of biochemical progression.[108,109] Gleason score are thought to be lower when TMPRSS:ERG is present.[110] No correlation was observed by other five studies that compromised similar sized study cohorts.[106,111-114] Also the presence of ETV1 rearrangements failed to correlate with progression of disease.[115] Most reports reveal an unfavorable correlation of gene rearrangements with outcome after treatment (radical prostatectomy). These studies showed an increased rate of biochemical recurrence, formation of metastases or even death.[114,116-124] Interestingly, one study showed that ERG rearrangement alone was associated with low grade prostate cancer, present with seminal vesicle invasion there seemed to be a poorer prognosis.[105,122] Expression of the TMPRSS2:ERG fusion gene was shown not to be able to predict response to endocrine treatment in hormone dependent and lymph node positive prostate cancer.[125,126]

Rearrangements of genes from the ETS family are potentially very useful diagnostic markers due to their prostate cancer specific occurrence if they can be measured in serum or urine. Like for PCA3, a test has been developed to measure fusion transcripts in

urine. For prognostic or predictive purposes, fusion gene-based tumor markers remain controversial.

Because measurements of the fusion transcripts and genes are performed with RT-PCR or FISH (fluorescent in situ hybridization) techniques, implementation in daily clinical practice is hampered. Recently, an antibody against the ERG protein was generated that can be used for immunohistochemistry.[127,128] Although the antibody has some cross-reactivity with FLI1, it gives the opportunity to easily and quickly assess thousands of retrospective and prospective patient samples. All three techniques (ERG antibody on protein level, RT-PCR on mRNA level and FISH on DNA level) provide their own unique information on the status of the fusion event and are likely complementary in their diagnostic and prognostic value.

AMACR

AMACR (alpha-methylacyl coenzyme A racemase) is an enzyme that is encoded by the P504S/AMACR gene. In cells, this protein is located in the mitochondria and peroxisomes and although the function has not been revealed completely it is related to the metabolization of fatty acids and bile acid biosynthesis.[129-131] The AMACR transcript and protein are known to be highly expressed in a variety of cancers with a very high (up to nine times higher) expression in 86% off all prostate cancers.[132-134] In 2002, AMACR was introduced as a new marker for prostate cancer.[135] A meta-analysis of multiple mRNA expression arrays revealed that AMACR is over expressed in prostate cancer with high sensitivity and specificity.[136,137]

In a diagnostic setting, the use of the AMACR protein on immunohistochemical analysis of prostate biopsy samples has been limited to a valuable complement to other known markers.[138] Unfortunately, samples that did not contain prostate cancer also had AMACR expression, but generally lower compared to the cancer samples.[139] In 18% of the prostate cancers, AMACR is false negative.[140] When unusual histopathological subgroups of prostate cancer had to be identified, the increased expression was only limited to 62-77%.[132,141]

In a prognostic setting it has been shown that untreated metastasis and hormone-refractory prostate cancers were strongly positive for AMACR. In this specific prostate cancer stages, AMACR has a sensitivity of 97% and a specificity of 92-100%.[135,142] Furthermore, decreased expression of AMACR has been shown to have prognostic value in predicting biochemical recurrence and prostate cancer related death.[143]

In order to assess this marker in non-invasive derived patient materials (not biopsies) such as serum or urine, expression of AMACR mRNA could also be identified in 69% of the cases. Unfortunately, AMACR is not specific to cancer of the prostate, because

serum levels can also be elevated in other urological disorders like BPH or auto-immune diseases.[144] When used in a diagnostic setting as an additive to PSA, sensitivity and specificity can be increased when measured in urine, especially when the PSA is in the midrange (4-10 ng/mL).[145-147] Unfortunately, when AMACR mRNA was normalized to PSA mRNA, AMACR did not accomplish to be a statistically significant predictor of prostate cancer.[148] New promising serum tests for prostate cancer which comprehend the AMACR gene are evaluated. With these tests a ratio is calculated between the expression of the AMACR gene and the PSA gene.[131] Until now, one report has been published were it was shown that the AMACR protein is detectable in serum with an ELISA, but elevation of this protein was not specific for prostate cancer.[149] Although more research has to be performed, it is also shown that circulating antibodies against the AMACR protein in combination with PSA could function as a useful tool for diagnosis.[146,150]

GSTP1

During aging, DNA damage occurs as a result of oxidative stress, exposure to chemical substances or ionizing radiation.[151] These damages can result in mutations or alterations of oncogenes and tumor suppressor genes. In healthy cells the cytoplasmic enzyme glutathione S-transferase pi I (GSTP1) plays an important role in detoxifying the cell from carcinogens. GSTP1 is a member of the glutathione S-transferase family, which contains four different classes. All these classes are expressed in prostate tissue.[152] Although GSTP1 expression is increased in various cancers, in prostate cancer GSTP1 is down regulated.[153] This is caused by hypermethylation of the GSTP1 promoter, a mechanism well known in cancer to decrease expression of tumor suppressor genes. Hypermethylation of GSTP1 was observed in all stage of prostate cancer, from high grade PIN to metastases.[154,155] Such methylation was not observed in benign prostate epithelial cells.[151] Based on these findings and the presence of methylation in 90% of prostate cancers and 67% in high grade PIN, it was concluded that GSTP1 methylation might function as a tumor marker for prostate cancer.[156,157] Subsequently, methylation of this gene could be observed in serum, urine and ejaculate of prostate cancer patients when analyzed by methylation specific PCR, which gave rise to the idea that it could even be applied in a clinical setting.[158-161]

As a diagnostic marker it was shown that GSTP1 DNA methylation in urine has a sensitivity of 75% (after DRE) and a specificity of 98% for prostate cancer and is comparable to its expression in biopsy specimen.[162] Similar values for sensitivity and specificity were observed in other studies. It is notable that sensitivity in urine is increased by collection directly after digital rectal exam or prostate massage and functions independent of

PSA.[163-165] To increase sensitivity even more, a relative ratio of GSTP1 methylation over methylated MYOD6 can be determined.[153]

For prognostic purposes, 100% of the locally advanced or metastatic tumors showed hypermethylation. Biochemical recurrence after prostatectomy seems to appear more and faster when the epigenetic alteration is present.[166] In a small study cohort it was shown that methylation of GSTP1 is a statistically significant predictor for time to recurrence.[167] Androgen deprivation therapy does not seem to influence GSTP1 methylation in 87% of the cases.[168] Unlike other genetic alterations, methylation of this gene is reversible after therapeutic intervention. Because no reports have been published which describe this effect, more research is needed.

Methylation of GSTP1 seems to function very well as a diagnostic and prognostic tool, but because the number of reports describing this marker is lacking, we should be careful in jumping to conclusions. As more results are being published, more allusions are made regarding the use of a set of hypermethylated genes for optimal diagnosis and determining prognosis in prostate cancer patients.

PSMA

PSMA (Prostate specific membrane antigen), or also known as FOLH1, is a androgen regulated gene that encodes a type II transmembrane glycoprotein. PSMA belongs to the M28 peptidase family and has a intracellular and extracellular domain.[169] Its function is limited to hydrolyzing peptides in prostatic fluid and generating glutamate and also acts as a folate hydrolase.[170,171] This protein is expressed in a number of tissues such as prostate, nervous system and kidney.[172,173] Furthermore, it has been shown to have a higher expression in prostate cancer. This finding could possibly be related to its enzymatic activity and thus invasiveness growth of prostate cancer.[174,175]

In the field of prostate cancer, PSMA has been the focus of many research groups. It has mainly been suggested as a prognostic tool.[176] Immunohistochemical analysis in a group of 232 patients showed higher expression in prostate cancer (79.3%) and metastases (76.4%) as compared to benign prostate tissue (46.2%).[177] Other studies showed an increased expression in progressive prostate cancer and hormone independent prostate cancer.[178-183] In serum from prostate cancer patients, the PSMA protein is increased, with a higher expression in advanced stages of cancer.[184-186] Nevertheless, contradicting studies show that PSMA is not prostate cancer specific and does not discriminate between localized prostate cancer and advanced disease.[187] A possible explanation for these different findings could be the fact that in those studies different types of antibodies have been used in various assays. Also studies that investigated the expression of PSMA mRNA have shown varying and inconclusive results, probably because of different assays used.

The sensitivity of diagnosing prostate cancer with PSMA mRNA is more or less similar to that of PSA mRNA.[174] As a prognostic marker no correlation was observed between PSMA mRNA and Gleason score, pT staging and serum PSA. In a study on patients with clinically localized prostate cancer, a combined PSMA/PSA mRNA analysis in peripheral blood samples showed that this could be an independent predictor to biochemical progression after radical prostatectomy.[188]

Although PSMA seems to be not prostate and prostate cancer specific, there is an upregulation of PSMA in prostate cancer and probably more in its aggressive forms. Therefore its function as a marker for prostate cancer is limited. A more promising feature of PSMA is its application in tissue targeted therapy such as prostate specific cancer vaccine therapy or radioimmunotherapy.[189,190]

PSCA

Prostate stem cell antigen (PSCA) is a gene that encodes for a membrane based glycoprotein. PSCA has been found to be relatively highly present in prostate, but also in other cell types such as bladder, placenta and gastrointestinal tissues.[191] The expression is also elevated in malignant tissues such as prostate cancer, bladder cancer and gastrointestinal cancers.[192,193] In prostate the expression of the PSCA mRNA is influenced by puberty, androgen deprivation and androgen restorement.[194] Although the exact involvement of PSCA in prostate cancer is fairly unknown it was shown that PSCA protein and mRNA are higher expressed from high grade PIN through all stage of prostate cancer.[195,196] Nevertheless, knockout of the PSCA gene in mice resulted in a normal urogenital development without an increased risk of prostate cancer.[197]

As a diagnostic or predictive marker it was shown that expression of PSCA in negative biopsies before TURP (transurethral resection of the prostate) is associated with higher risk of having prostate cancer in the TURP specimen. Especially when serum PSA levels >4.0 ng/mL or with a suspicious DRE.[198]

In a prognostic setting, immunohistochemical analysis showed that expression of the PSCA protein was present in 94% of all tumors and was significantly associated with adverse prognostic features, such as high Gleason score and extra-capsular extension.[199,200] Furthermore, PSCA was identified in bone metastases and lymph node metastases.[201,202] These findings suggest that there is a positive correlation of the PSCA protein with advancement of disease status in prostate cancer. When PSCA mRNA was measured in peripheral blood it corresponded with a reduced disease free survival time.[203] Compared to PSA and PSMA it was noticed that specificity and independent prognostic value were very high.[203] Unfortunately this transcript could only be identified in 13.8% of the patients, which limited its ability to differentiate between benign and malignant prostate

tissue. When this marker was investigated for its post-treatment monitoring value, it was shown that after EBRT PSCA mRNA is decreased.[204] Therefore it was proposed as in interesting marker for follow-up after treatment.

Besides the properties of being a possible diagnostic or prognostic marker for prostate cancer, it has also been found that PSCA is a possible target for prostate specific virus therapy.[205,206] When PSCA is used, it was possible to inhibit tumor growth and formation of metastases.

CHROMOGRANIN A

Chromogranin A (CgA), is a gene that encodes for a proteolytic protein that is a member of the chromogranin/secretogranin family of neuroendocrine secretory proteins. CgA is one of the most frequently produced proteins in neuroendocrine cells in the prostate and can be easily measured by a radioimmunoassays.[207] Serum levels of Chromogranin A could reflect neuroendocrine activity of prostate malignancies, therefore it holds an interesting potential to function as a marker for prostate cancer and especially for neuroendocrine differentiation.[208,209] Unfortunately, Chromogranin A is not prostate specific, it is also elevated in various neuroendocrine tumors and neuroblastomas.[210-213] The exact function of Chromogranin A in prostate cancer is unknown, but it has been shown that it influences the growth of prostate cancer cells.[214]

Despite conflicting results as a diagnostic tool, when measured in serum, high Chromogranin A levels seem to correspond with the presence of (organ confined) prostate cancer. [216] In combination with PSA a better diagnostic accuracy could be established.[215] An interesting report showed that Chromogranin A is able to predict conversion of hormone naïve prostate cancer to hormone refractory disease and the presence of hormone independent prostate cancer itself.[216,217] A small prospective study on 50 prostate cancer patients showed that high Chromogranin A serum levels prior to radical prostatectomy were able to predict higher Gleason scores, extra capsular extension and eventually treatment failure.[218-220] Especially in patients with hormone independent prostate cancer this marker correlates with adverse outcomes and decreased overall survival.[221] Furthermore, this marker could function as a predictor for chemotherapy response in hormone independent prostate cancer.[222] In a prognostic setting, high levels of CgA correspond with factors such as a higher Gleason score, advanced pT stage and metastases.[223,224] Immunohistochemical analysis showed similar results.[225,226] No decrease in Chromogranin A serum levels were observed after radiotherapy or hormone therapy, Therefore the use of this marker in as a monitoring tool seems not to be sefull.[227,228] Specific antibodies against Chromogranin A can suppress its function through apoptotic pathways, leading to programmed cell death. Therefore Chromogranin A antibody mediated apoptosis was

suggested as an alternative treatment for prostate cancer.[214] A derivate of this marker, Chromogranin A velocity was introduced as a marker for predicting time to androgen independence after hormonal treatment.[228]

B7-H3

The transmembrane protein family B7 has gained publicity with its role in regulation of T lymphocytes.[229] Subsequent reports showed that a total of four subtypes (B7-H1, B7-H2, B7-H3 and B7-H4) could be identified in cancers and might play a role in the mechanism by which human malignancies evade host immune responses.[230-232] Higher expression of some of these subtypes are correlated to more aggressive behavior and poor clinical outcome.[233,234] The B7-H3 has also been identified in healthy placenta and malignant tissues.[235] Although there was expression in benign tissue, the expression in cancerous lesions was significantly higher.[230]

B7-H3 could be identified as an independent prognostic factor in 338 patient samples after radical prostatectomy that were followed with a median of 3.9 years. The patients which showed elevated B7-H3 expression had a shorter time to cancer progression.[236] This indicated that B7-H3 could function as a prognostic marker. Furthermore, B7-H3 expression is higher in metastases and hormone refractory prostate cancer. The expression is not hampered by hormone treatment.[237] Also, this marker could have prognostic value for biochemical recurrence after salvage radiotherapy, especially with low primary TNM staging, low Gleason score and low pre-radiotherapy PSA.[238] Because this marker is membrane-bound in cells it also harbors a function in targeted therapy. Chemotherapy or radionucleotide therapy that is directed against B7-H3 makes it possible to specifically engage prostate cancer cells.

CAV1 (CAVEOLIN-1)

Caveolin-1, is a major structural component of caveolae. These caveolae are specialized membrane invaginations that are abundant in adipocytes, endothelium and smooth muscle cells. Caveolae are involved in molecular transport, but also in cell adhesion and signal transduction.[239,240] Caveolin-1 has been linked to prostate cancer since the late 90s, where it was identified as a marker.[241] The exact relation of caveolin-1 and prostate cancer remains unclear, but it is known that caveolin-1 in prostate acts as a tumor suppressor by keeping Akt dephosphorylated in the Akt-pathway.[242] Subsequently it was shown in *in vitro* experiments that downregulation of the expression of this gene resulted in cells turning from androgen-independent to androgen-dependent.[243] This

implicated that there is a role for Caveolin-1 in the development of castration resistance. It is also known that this protein plays a role in the malignant characteristics of prostate cancer cells by changing the microenvironment and promoting angiogenesis.[244] Studies showed that Caveolin-1 is also expressed in normal prostate stromal cells, but minimally expressed in normal epithelial cells.[245] The protein expression of Caveolin-1 is higher in prostate cancer cells compared to normal prostate epithelial cells.[241] The expression of this marker in epithelial cells upregulates when prostate cancer grading increases.[245] Furthermore, the protein Caveolin-1 also has higher serum values in patients with prostate cancer, which makes it possible to measure it with a very sensitive and reproducible ELISA.[246] Median serum Caveolin-1 levels are significantly higher in localized prostate cancer compared to men with BPH.

Caveolin-1 levels could harbor a predictive potential in men undergoing radical prostatectomy.[247] Higher expression of Caveolin-1 was correlated with an increased risk of developing aggressive recurrent tumors after surgical treatment. Pre-operative high Caveolin-1 serum levels resulted in a 2.7 fold higher risk of developing biochemical recurrence.[248]

When Caveolin-1 was investigated as a prognostic tool, in samples retrieved after radical prostatectomy it was shown that a positive immunohistochemical staining correlates with a significant worse prognosis.[249] In patients with lymph node negative prostate cancer, Caveolin-1 expression is an independent prognostic factor for a Gleason score >7, extra prostatic extension, positive surgical margins. When combined in a multivariate model with other variables such as Gleason score it is possible to more accurately predict the chance of biochemical recurrence. Unfortunately, another study showed in 1458 cases no correlation between high post-operative Caveolin-1 values in serum and aggressiveness of prostate cancer or adverse prostate cancer events.[250]

GOLPH2

GOLPH2 (Golgi phoshoprotein 2), also known as GOLM1 or GP73, is a type II Golgi membrane protein and involved in the sorting and modification of proteins that are exported from the endoplasmatic reticulum through the Golgi apparatus. Recent findings suggest that changes in structure and function of the Golgi apparatus may play an important role in the development or behavior of malignant cells. This protein has already been shown to be elevated in liver diseases as a result of viral infections, but also as a potential marker for hepatocellular carcinoma.[251,252] Immunohistochemical experiments on prostate cancer samples revealed that the GOLPH2 protein also is upregulated in prostate cancer.[253,254] An interesting finding was that this specific marker is present, even when AMACR is negative. Therefore it was mainly introduced as an additive protein

marker for prostate cancer, next to other known markers. Preceding mRNA profiling studies, research already showed that GOLPH2 mRNA is upregulated in prostate cancer tissues.[255,256] When this gene transcript is used in a marker profile to detect prostate cancer in urine, it seems to be capable to outperform PSA measured in serum.[148]

MYO6 (MYOSIN IV)

Myosin IV is a Golgi apparatus-associated protein that is involved in intracellular vesicle and organelle transport and is required for the structural integrity of the Golgi apparatus. Furthermore the protein has been suggested as an important factor for cell migration and even cancer invasion.[257-259] Based on a microarray experiment it was discovered that the MYO6 mRNA is upregulated in prostate cancer, next to GOLPH2.[260] Interestingly, expression of the transcript goes down in androgen-independent and more aggressive prostate cancers.[260] With Immunohistochemical analysis it was shown that a strong protein expression is present in a PIN, the majority of prostate cancer cells, and weak or absent expression in neighboring benign prostate cells.[254]. In a prognostic setting, no differences were observed between the different Gleason scores or other pathological indicators for aggressiveness.[260] Based on these results, the transcript could be used as a diagnostic marker, but further research has to be performed to reveal the true potential of this marker and to assess its possible role in prognosis.

CRISP3

Cysteine-rich secretory protein 3 (CRISP3), also known as specific granule protein 28 (SGP28), has recently been implicated as potential marker in prostate cancer. Relatively little is known about its function and role in prostate cancer. The CRISP3 mRNA has shown to be present in high concentrations in salivary glands, pancreas and prostate.[260-262] Furthermore, its expression has been shown in secretory epithelium in the male urogenital tract, including the epididymis and the ampullae of the ductus deferens.[263] Regarding prostate cancer, multiple studies have shown that the expression of the CRISP3 mRNA is high[264]er (20-300 times) in prostate cancer as compared to healthy prostate tissue.[262,265,266] Also on the protein level, CRISP3 was shown to be higher expressed.[267] The protein also has been identified by ELISA in multiple bodily fluids, such as serum, saliva and seminal plasma.[268] Unfortunately, serum concentrations were not different between prostate cancer samples and healthy controls.

As a prognostic marker, immunohistochemical analysis of prostate cancer specimen showed an increase in expression when Gleason scores increased. Expression in normal

prostate epithelial cells was weak of absent. A similar analysis on radical prostatectomy samples revealed that expression of CRISP3 eventually positively correlated with biochemical recurrence.[269] In a multivariate analysis this protein was still associated with recurrence. Nevertheless, when this marker was added in a model with other known markers, such as PSA, no improvement was observed. With the results acquired so far, CRISP3 does not seem to be a good prognostic marker for prostate cancer.[264,270]

An interesting observation was the decrease of CRISP3 after ochiectomy in some patient samples. This could reflect that CRISP3 could be partially androgen regulated and might function as a monitoring marker.

SARCOSINE

The discovery of Sarcosine as a marker for prostate cancer has only recently been made. Since a large number of research groups are exploring changes on the level of genomics, transcriptomics and proteomics, changes in the metabolomic field are novel and few. Sarcosine is a metabolite that is produced by the enzymatic transfer of a methyl group from S-adenosylmethionine to glycine. This reaction is catalyzed by the enzyme glycine-N-methyltransferase (GNMT), which is highly expressed in prostate, liver and pancreas. The first report on Sarcosine in prostate cancer showed that Sarcosine stimulates malignant growth of prostate cancer cells and has prognostic value.[271] With mass spectrometry they analyzed blood, urine and tissue samples from different well characterized prostate cancer patients and explored them for metabolites. In a relatively small patient cohort a total of 1126 metabolites were identified. Sarcosine was highly increased during prostate cancer progression to metastasis and could easily be identified in urine.[271] Subsequently they showed a decrease in disease progression when glycin-N-methyltransferase was knocked down.

Although these results look very promising, subsequent reports showed that Sarcosine as prognostic marker is debatable. On tissue samples the expression in cancerous samples was 7% higher compared to benign prostate samples. Unfortunately no statistical differences were seen regarding prostate cancer progression.[272] A drawback of this study was the fact that metastatic samples were not included. Also Sarcosine as a urine marker, normalized to creatinine, could not reproduce the original finding that Sarcosine functions as a prognostic marker. [273] When compared to PSA, urine derived Sarcosine was not able to outperform serum PSA on itself. When added to an algorithm with PCA3 or %fPSA diagnostic performances could be improved.[274]

Although Sarcosine was promoted as a promising new marker for prostate cancer, its exact clinical value and applicability is unclear. The conflicting reports are mostly based on a limited number of samples with limited follow-up and different technologies to

measure this metabolite. In order to resolve these contradictions we need to control some of the variables, such as the study cohort and tumor marker assays.[275]

EXOSOMES

Exosomes are small vesicles (50-150 nm) that are shed by almost all cell types in the human body into almost all body fluids. Initially, exosomes were discovered during studies on the loss of the transferrin receptor loss in sheep reticulocyte maturation.[276] Exosomes are formed by inward budding of the cellular membrane which results in the formation of a large endosome. After formation of the endosome it is subjected to a second step of inward budding. During this second step, cytoplasmic content is taken up in small vesicles. When the endosome (now referred to as multivesicular body) is filled with small vesicles it fuses with the cellular membrane and the small vesicles, or so-called exosomes, are shed in the extracellular space.[277,278] Because of this biogenesis pathway, exosomes contain proteins and RNA that are specific for the cell from which they are derived and thus represent the state of the cell.[279] By isolating prostate (cancer) derived exosomes one is able to search for new and specific tumor markers for prostate cancer. The reports on exosomes in prostate cancer are limited. One of the first clinically related studies showed their potential. The quantity of exosomes isolated from urine is higher in prostate cancer patients as compared to healthy controls.[280] Unfortunately, in this study nothing was reported about differences in exosomal content. RNA expression analysis revealed that known markers of prostate cancer such as the TMPRSS2:ERG fusion mRNA and PSA mRNA could be identified in exosomes.[279] This finding emphasizes their function as tumor marker containing structures.[277,281]

Although the reports are limited, the study populations are very small and the variation in number of exosomes, exosome research in prostate cancer could accelerate tumor marker discovery. Because they are present in body fluids, noninvasive technique can be applied to isolate exosomes and use them for diagnose or monitor the course of prostate cancer.[282] Unfortunately, when isolating exosomes from serum or urine no distinction can be made between the different tissues from which the exosomes are derived. Therefore more research has to be done to specifically isolate and profile prostate (cancer) derived exosomes.

SUMMARY

Currently, PSA is the best and most widely accepted prostate tumor diagnostic and monitoring marker we have available for daily medical practice. Nevertheless, its limita-

tions cause a need for new and more accurate markers. From the many discovery endeavors, there seems to be an inexhaustible source of new potential tumor markers that are being explored. Unfortunately, most of these candidate tumor markers still need to be evaluated more thoroughly to validate their diagnostic or prognostic value and demonstrate their added value over current practice.

Because of the heterogeneity of prostate cancer there is a fairly good chance that the use of single tumor marker will not cover all aspects of the disease and a combination of two or more markers is needed. In addition, multiple markers will be needed to address the different types of relevant clinical decision points, ranging from risk assessment, diagnosis and personalized therapy.[283] Importantly, different technologies including mass spectrometry and microarrays are being introduced into the clinical to measure novel markers and extend the types of markers from the typical proteins to metabolites, DNA and RNA.

Despite the large efforts invested in prostate cancer marker research in the past decade, the number of clinically valuable markers is very limited. We have learned that open and unselective searches in a discovery phase, generally result in many new candidate markers, but also that most of these are not validated in independent and larger cohorts. It has become painfully clear that the complexity of body fluids and tissues, a selection bias and inadequate number of samples for discovery and the variation between individuals are some of the major hurdles in the ongoing quest for novel markers. Despite these challenges, more accurate and reproducible technologies, more focused explorations and the growing number of samples in (consortium) tissue banks, improve the essential steps of excluding false positive candidates in an early stage and robustly validate novel markers.

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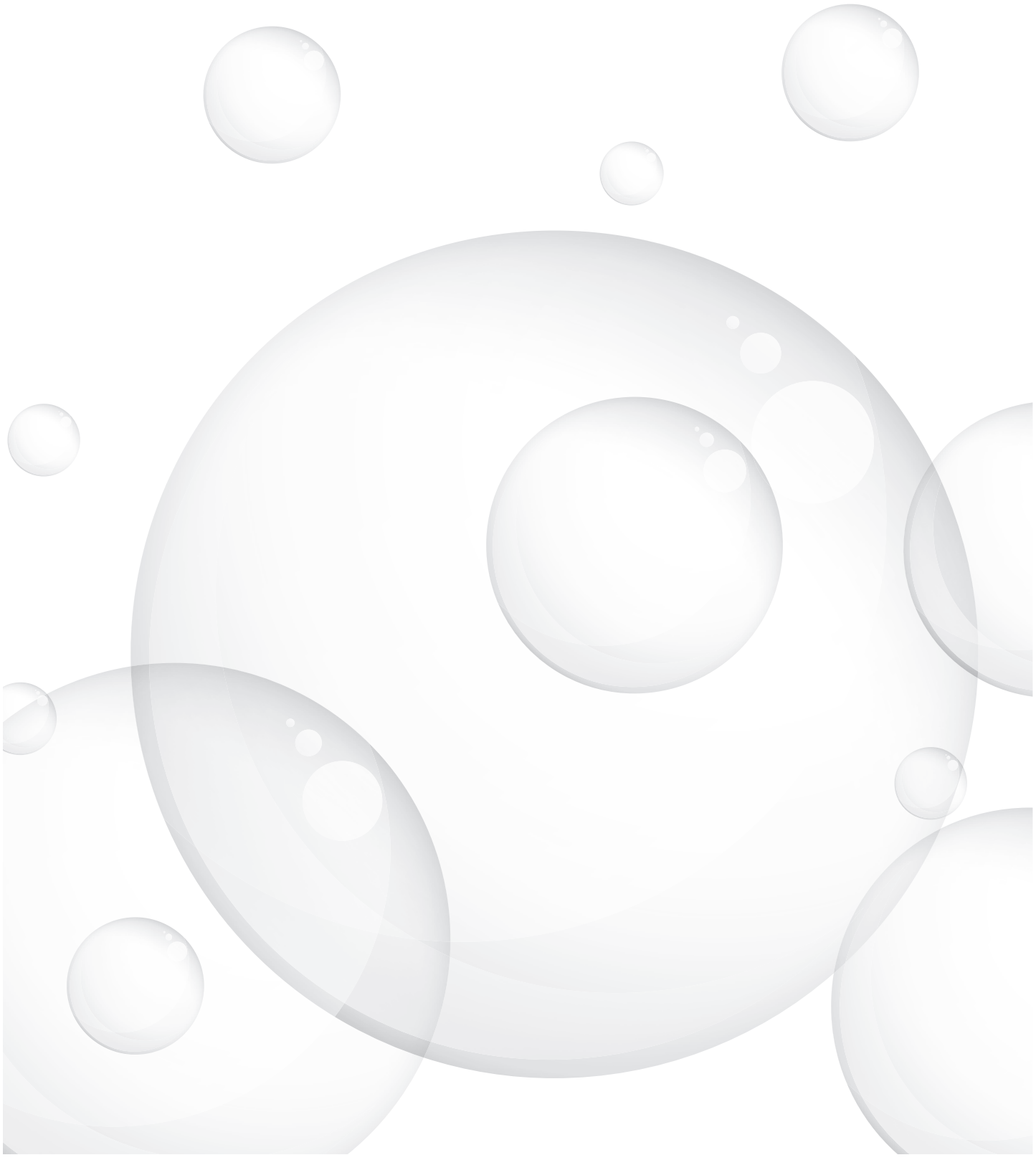
PART 1

Extracellular vesicles
and biomarker discovery



3

Exosomes as biomarker treasure chests

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ABSTRACT

Context

Although progress has been made in respect of types of markers (protein, DNA, RNA and metabolites) and the implementation of improved technologies (mass spectrometry, arrays, and deep sequencing), the discovery of novel biomarkers for prostate cancer (PCa) in complex fluids, such as serum and urine, remains a challenge. Meanwhile, recent studies have reported that many cancer-derived proteins and RNAs are secreted through small vesicles, known as exosomes.

Objective

This narrative review described recent progress in exosome research, particularly focusing on their potential role as novel biomarkers for PCa. The purpose of this review was to acquaint the clinicians and researchers in the field of urology with the potential role of exosomes as biomarker treasure chests and their clinical value.

Evidence acquisition and synthesis

Medline and Embase entries between 1966 and September 2010 were searched using the key words of exosomes, microvesicles, prostasomes, biomarkers, prostate cancer and urology. Leading publications and articles constructively contributing to exosome research were selected for this review.

Conclusions

Exosomes are small vesicles (50 to 100 nm) secreted by almost all tissues; they represent their tissue origin. Purification of prostate (cancer)-derived exosomes will allow us to profile exosomes, providing a promising source of protein and RNA biomarkers for PCa. This profiling will contribute to the discovery of novel markers for the early diagnosis and reliable prognosis of PCa. Although the initial results are promising, further investigations are required to assess the clinical value of these exosomes in PCa.

INTRODUCTION

Prostate cancer (PCa) is one of the few solid tumors with a clinically useful biomarker for both diagnostics and follow-up after treatment. This biomarker protein, prostate-specific antigen (PSA), has been considered the "gold standard" for the detection of PCa.[1] Although PSA has acceptable sensitivity, it lacks the specificity for discriminating benign prostate diseases (*e.g.,* benign prostatic hyperplasia (BPH) and infection), indolent PCa and aggressive PCa. It has also been shown that PSA-based screening leads to a decrease in the prevalence of advanced PCa and a reduction of PCa-related mortality by 20%.[2,3] However, this screening is also associated with a high risk of overdiagnosis and over-treatment based on findings on complementary diagnostic prostate biopsies. Therefore, new molecular markers for PCa are needed to more specific, to prevent unnecessary prostate biopsies and to help the urologists to decide the most optimal treatment.[4]

Searching for novel biomarkers has been the focus of many research groups, and the studies have become more extensive and sophisticated. Although exciting progress has been made in respect of novel technologies, such as mass spectrometry analysis or RNA-based arrays, discovering new biomarkers in serum and urine remains a challenge. Particularly, proteomic profiling from complex body fluids is hampered by several problems. One of these problems is that a few high-abundance proteins (albumin, immunoglobulins, transferrin, complement factors, fibrinogen, and so on) make out 97% of body fluids, whereas low-abundance proteins are generally the most promising candidates for biomarker discovery.[5] As indicated, this dynamic range of protein concentrations is very large (*e.g.,* serum contains 7.5×10^5 nmol/L albumin and 10^{-1} nmol/L PSA (3 ng/ml), meaning that for every single molecule of PSA, 7.5 million molecules of Albumin are present. Mass spectrometry has made large-scale proteomics analysis feasible; however, the high-abundance proteins reduce the detection sensitivity of this technology.[6] Most likely, promising marker proteins are probably present at the concentration of 10^{-3} to 10^{-5} nmol/L. The sensitivity of mass spectrometry has a detection limit of up to 10^2 nmol/L.[7] Due to the dynamic range issue, identification and quantification of the low-abundance proteins remains a great challenge. Therefore, even with current "state-of-the-art" technologies, discovering novel biomarkers is still like searching for a needle in a haystack.

This dynamic range problem can be partially tackled by several methodologies. For example, high-abundance proteins can be depleted by chromatography or by precipitation. Moreover, fractionating the samples into many different portions, for instance, by isoelectric focusing, mass separation or affinity chromatography, can improve the identification of low-abundance markers. Unfortunately, fractionation increases the number of measurements and, consequently, the time to process an individual sample. The detection sensitivity can increase approximately 100-fold by combining these two methods; however, it is still not enough to identify the low-abundance markers.[8]

Another option that may contribute to the better identification and detection is specific enrichment. An obvious problem with this approach is that in a discovery setting, it is not known what the protein or RNA marker of interest is. However, recent findings have revealed that small tissue-derived vesicles, the so-called exosomes, are present in serum and urine and contain a wide range of proteins and RNAs [9,10] that represent their tissue origin. These vesicles also express tissue-specific transmembrane proteins that can be used for specific isolation of the vesicles from the complex fluids. Enrichment of cancer-derived vesicles from complex body fluids may solve the dynamic range problem and allow the identification of novel biomarkers.

OBJECTIVE

Since the last decade, exosome research has been rapidly expanded, and the number of coherent publications has been gradually increasing. Therefore, it is necessary to acquaint the clinicians and researchers in the field of urology with this biological concept. The main objective of this narrative review was to describe recent progress in exosome research, especially in the field of urology, particularly focusing on their potential role as novel biomarkers for PCa.

EVIDENCE ACQUISITION AND SYNTHESIS

All entries between 1966 and September 2010 in Medline and Embase were searched to identify original studies and review articles. Leading publications and original articles constructively contributing to exosome research were included. For focusing the exosome research in the field of urology, the search was conducted using the following key words: (exosome* OR microvesicle*) AND (prostate cancer OR urology). The search was limited to the publications written in English with the full text available. Initially, we reviewed titles and abstracts for clinical relevance. A total of 25 manuscripts were reviewed, from which five were selected. Because the term of exosome has also been used in literature for a RNA-degradation complex, we manually excluded the articles describing such complex to prevent confusion.

Biogenesis and secretion of small vesicles

Exosomes (50 to 150 nm in diameter) were first described in sheep reticulocyte maturation in 1983.[11] In studies on transferrin receptor loss during reticulocyte development, it has been noticed that this plasma membrane receptor is shed through small vesicles.[12,13] The biogenesis of these vesicles starts from the internalisation of cellular membrane that,

thereby, forms an early endosome. During the formation of this endosome, cytoplasmic content is taken up by inward budding of endosomal membranes, resulting in exosome formation. When exosomes are formed, the endosome is called a multivesicular body (MVB). When the MVB fuses with the cellular membrane, the vesicles are secreted (Figure 1).[14]

The exact mechanisms involved in exosome biogenesis are not fully elucidated; however, some factors have been reported to play a role. First, specific lipids and transmembrane proteins are grouped in the cellular membrane.[15] These groups form separate microdomains, the so-called lipid rafts. These lipid rafts are enriched with glycosphingolipids and contain transmembrane cross-linked proteins.[15,16] Although the exact role of lipid rafts in exosome formation is not clear, they seem to exert an important regulatory effect. Second, for sorting and encapsulating cellular content into exosomes, protein complexes, such as "endosomal sorting complex responsible for transport (ESCRT)", and the process of protein ubiquitination are involved.[17] The function of these protein complexes is regulated by Vps4.[18] Third, exosome secretion is partially regulated by multiple Rab proteins, which control intracellular transport pathways by regulating vesicular trafficking. Especially, Rab27A, Rab27B and Rab35 have been shown to be important regulators in vesicle secretion.[19]

Although we only partially understand biogenesis of exosomes, we do know that they contain cytoplasmic content (proteins and RNAs) that is encapsulated by a cholesterol-rich phospholipid membrane consisting of a host of transmembrane proteins.[20,21] Exosomes probably represent the transmembrane and intracellular conditions of their cell origin. Furthermore, the process of the biogenesis and shedding of exosomes has been shown in many mammalian cell types, including malignant cells; it is an independent pathway, compared to the secretion of signal peptide proteins (such as PSA) that are processed through the classic consecutive route (Figure 1). Therefore, profiling the exosomes derived from specific tissues may contribute to the understanding of the pathogenesis of tissue-related diseases.

Exosomes and their functions

Exosome shedding is a process with a wide range of important regulatory functions. Their discovery in sheep reticulocyte maturation gave rise to the idea that exosomes may function as a trash bin for unnecessary and redundant proteins. Therefore, it could be an alternative pathway for lysosomal degradation.[22] Nevertheless, most attention has been paid to their role in the immune system. Functional experiments have shown that exosomes affect the immune system by expressing and processing antigens.[23] First of all, exosomes are enriched with specific antigens, compared to whole cell lysates.[24] Second, exosomes from antigen-presenting cells (APC) contain large amounts of major histocompatibility complex (MHC) class I and II molecules.[21,25] When APC-derived exo-

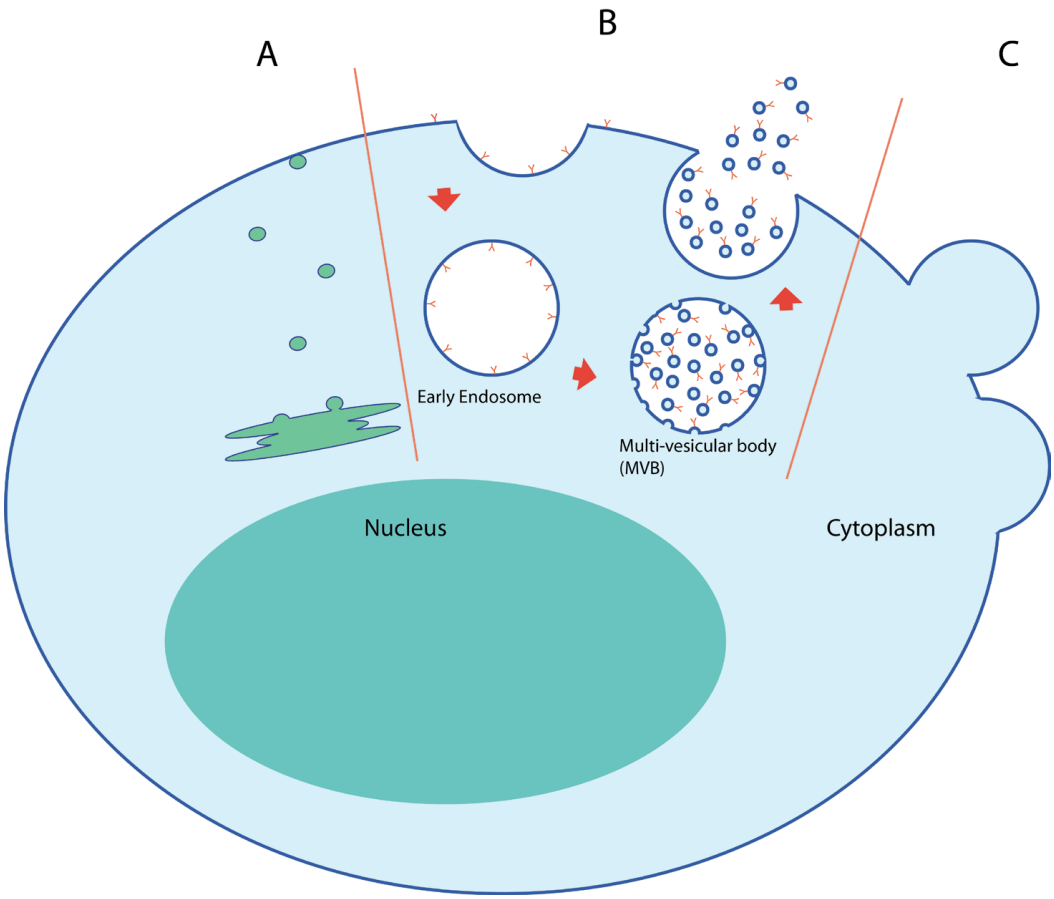

Figure 1. Schematic overview of different secretion mechanisms. (A) Secretion of signal peptide proteins through the classic consecutive route and processed through the rough endoplasmic reticulum and Golgi. (B) Merocrine secretion pathway. Cellular membrane buds inward and forms an early endosome. After the formation of such an endosome, cytoplasmic content is internalised into small vesicles (100 nm), the so-called exosomes. When exosomes are present inside the endosome, the endosome forms a multivesicular body (MVB). The MVB fuses with the cellular membrane, and the exosomes are released. (C) Apocrine secretion pathway. Proportionately larger vesicles (500 to 1000 nm), such as oncosomes, are formed by membrane shedding.

somes are incubated with donor cells, MHC could be re-expressed in these cells.[26] These results indicate that there is an exchange of membranes or membrane proteins between exosomes and cells, and that, exosomes, therefore, harbour a communicative function. Aside from the membrane transfer, exosome content, such as proteins and RNAs, can also be shuttled between cells through exosomes.[27] By transferring RNAs, exosomes are capable of transferring genetic information that can be translated into functional proteins in target cells.[28]

In the field of cancer research, there is an ongoing debate regarding their exact role as pro- or anti-tumor effectors. Experiments in mice have shown that cancer-derived exosomes can induce protective anti-tumor immune responses.[29,30] It has been demon-

strated that exosomes isolated from malignant effusions are an effective source of tumor antigens to be presented to CD8+ cytotoxic T cells.[31] *In vitro*-derived exosomes can even function as cell-free vaccines and lead to a decrease or stabilisation of tumor growth.[32] A possible explanation might be that exosomes initiate immune-mediated cell death. Nevertheless, these scenarios look promising *in vitro* under stress conditions [33] but do not seem to be well-applied to clinical settings.[24] Opposite to the anti-tumor responses are other *in vitro* studies reporting a potential role of promoting tumor cell growth.[34] Particularly, the transfer of miRNAs through glioblastoma exosomes may induce tumor growth in a benign cell line.[35] Because the effects and content of exosomes can be versatile, it is not surprising that both pro- or anti-tumor effects have been described and that the role of exosomes might change during cancer progression.

Exosomes, prostasomes and other vesicles

Many types of vesicles have been described in literature. These vesicles are heterogeneous in terms of size, content and origin; therefore, they have different names. Unfortunately, the differences in nomenclature lead to confusion. It is still unclear if all of the different vesicles are unique in biological function, or if they represent a sliding scale of one entity. Based on their biogenesis, however, vesicles could be generally divided into two classes: merocrine (inward budding and exocytosis, such as exosomes) and apocrine (surface shedding) synthesis (Table 1).

Table 1. Characteristics of different types of vesicles secreted by prostate or PCa cells.[33,45]

Vesicle	Size (nm)	Known protein markers	RNA marker examples	Synthesis pathway	Function	Reference
Exosomes	50 - 150	CD9, CD63, CD81, CD82, Annexins, and RAB proteins	PCA-3, TMPRSS2:ERG	Merocrine	Antigen presentation, immune regulatory, and metastatic activity	10,45-47
Prostasomes	50 - 500	CD13,CD46, CD55, CD59, Annexins, and RAB proteins	-	Merocrine and apocrine	Immunosuppressive and sperm cell motility improving	36-39
Oncosomes	50 - 500	Signal transduction proteins	DIAPH3	Apocrine	ND	48
Microvesicles	100 - 1000	Integrins, selectins, and CD40 ligand	EGFRvIII	Apocrine	Procoagulation and anticoagulation	35,49
Ectosomes (microparticles)	50 - 1000	CR1 and proteolytic enzymes	-	Apocrine	Procoagulation and anticoagulation	50,51

ND: Not Defined

Studies on small vesicles in the field of urology mainly used the terms of prostasomes and exosomes. Usually, the vesicles isolated from seminal/prostatic fluids are called prostasomes. Confusion starts with vesicles isolated from prostate (cancer) cells cul-

tured *in vitro* or grafted in mice. Currently, both prostasomes and exosomes are used. Therefore, the questions are whether prostasomes are prostate-derived exosomes, and whether the prostasomes from seminal fluid are the same as the vesicles secreted by cultured cells.

Prostasomes have a pure prostatic gland origin and are present in high concentrations in seminal/prostatic fluid.[36] These vesicles are suggested to be shed through exocytosis after their formation in a MVB (merocrine), such as exosomes, and possibly also by membrane shedding (apocrine).[36] Compared to exosomes, they are enriched with cholesterol, sphingomyelin, Ca^{2+}, GDP and many transmembrane proteins (CD13, CD46, CD55 and CD59).[37-39]

Electron microscopy (EM) showed that prostasomes are round and have a mean diameter of 150 nm (50 to 500 nm). This description is highly similar to the exosomes derived from prostate epithelial cells that are also round-shaped and have a diameter of 100 nm (50 to 200 nm).[10] A striking difference is that exosomes usually contain a lipid bilayer membrane, whereas prostasomes usually contain a cholesterol-rich lipid multilayer membrane.[40] In terms of their functions, prostasomes have mainly been implicated in human reproduction by exhibiting a specific and favourable effect on the motility of spermatozoa [41] and by delaying acrosomal reaction.[42] Potential immunosuppressive activities of prostasomes have been demonstrated and are suggested to protect spermatozoa from phagocytosis by cells of the female immune system.[43] The protein content of prostasomes is comparable to that of the exosomes derived from prostate cancer cell lines. Most of the identified proteins are well-characterised intracellular proteins, including annexins, Rab proteins, heat shock proteins 70 and 90 (HSP70/HSP90) and signal transduction proteins.[43,44] The identification of biomarkers for PCa, such as PSMA (FOLH1) that is also present in exosomes, suggests that prostasomes may be a valuable source for novel biomarkers. So far, no reports have been published assessing prostasomal RNAs.

According to their marginal differences in size, morphology and content, the two types of vesicles are similar. Only their functions and potentially their lipid composition differ. Nevertheless, we hypothesised that prostasomes are exosomes derived from prostate tissue in a biological setting. Experimental comparison between exosomes and prostasomes may help differentiate their specific prostasomal properties from the more general characteristics and clarify their similarities and differences in biogenesis, content and function.

Isolation and visualisation of exosomes

Isolation

For morphological and biochemical characterisation, exosomes are usually isolated by differential ultracentrifugation. This well-developed isolation method has been shown

to be effective and can process up to 250 ml of samples. Unfortunately, this method is time-consuming (approximately 6 hours) and, therefore, is unsuitable for daily clinical practice and might affect RNA and protein quality due to degradation. Therefore, faster and simpler isolation methods, such as filtration, precipitation and immunoaffinity purification, are needed. Filtration techniques have already been established and can rapidly enrich exosomes from complex fluids.[47,52-55] Because it is particularly useful for smaller volumes, it could be easily implemented in a clinical setting.

When isolating exosomes from body fluids, it is impossible to distinguish exosomes derived from different tissues, which is a problem when searching for content in a subset of exosomes derived from a specific tissue or cell type. In this situation, immunoaffinity purification using beads or columns coated with an antibody directed against a tissue-specific transmembrane protein can be applied.[56, and unpublished work]

Visualisation

Because of the small size of exosomes, EM is the most suitable technique for morphological characterisation (Figure 2).[48,57] Using gold-labelled immune electron microscopy, it is possible to investigate whether exosomes express certain proteins on their membrane.[58] Another way of visualisation is to use confocal microscopy (CM), with the membrane of exosomes fluorescently labelled.[59] Although unlabelled exosomes are too small to be visualised by standard confocal microscopy, the lipophilic fluorescent dyes in their membranes is easily detected. Also, CM can be used to visualise exosomes isolated by

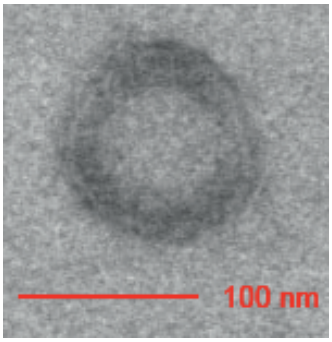

Figure 2. Electron microscopic image of an exosome. Close-up view of an exosome derived from the PC346c cell line. The diameter of the vesicle is approximately 100 nm, and the membrane is a lipid bilayer.

antibody-covered magnetic beads (Figure 3). Another advantage of this technique is the possibility of studying the functions of exosomes and their interaction with host cells.[60]

Quantification of exosomes

Counting exosomes in a sample remains a challenge. The number of exosomes is generally estimated by measuring the amount of protein.[47] The technology of fluorescence-activated cell sorting (FACS) is capable of counting exosomes; however, individually measuring each exosome (relatively tiny compared to a cell) in a flow system is difficult

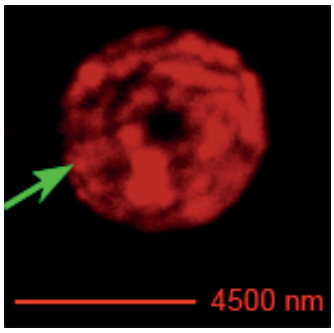

Figure 3. Exosomes attached to an immunoaffinity bead and visualised by confocal microscopy. These 4,500-nm size beads with antibodies directed against the exosomal membrane protein CD9 are incubated with fluorescently labelled exosomes derived from urine (100 nm). This close-up view of one bead shows that it contains multiple (red) exosomes. The green mark indicates a single exosome attached to the bead.

due to the resolution of the laser. These visualisation techniques (EM and CM) are not quantitative for determining the exact number of exosomes in a fraction but can be used to examine morphology and to determine transmembrane properties.

One study reported the successful use of a sandwich ELISA assay for exosome quantification.[61] In this assay, two different transmembrane proteins present on all exosomes are used. In theory, when such an assay is developed using one general transmembrane (capture) protein and one tissue- or cancer-specific transmembrane (detection) protein, the number of exosomes derived from a specific tissue can be measured.

Exosomes as biomarker treasure chests

The molecular content of exosomes is dependent on their cell origin and strongly associates with the original cellular conditions.[62] Therefore, the identification of tissue- or disease-specific exosomal proteins and RNAs will enable us to use these vesicles as a source of new biomarkers. Since the late 90s of last century, an increasing number of studies have investigated the protein content of exosomes and their potential diagnostic and prognostic values in various types of cancer, resulting in a comprehensive database consisting of 64 papers and a total of 2,400 different proteins.[63] All of these protein identifications have been obtained by mass spectrometry. In terms of RNAs, the first study on exosomes was performed in 2007.[28] Using microarray technology, they have shown that exosomes from mouse-derived bone marrow cells contain mRNAs and miRNAs. An increasingly number of papers, using microarrays as well, have described the potential role of proteins and miRNAs as diagnostic and prognostic tools.[64-66]

Until 2002, exosomes had been predominantly isolated and analysed from *in vitro* cell lines. More recent studies have showed that these vesicles can be isolated from body fluids, such as blood, urine, semen, amniotic fluid, malignant and pleural effusions, bronchoalveolar fluid, synovial fluid, saliva, and breast milk. These findings demonstrate that exosomes are present in all body fluids and can be used for determining health status.[67]

Exosomes as a diagnostic and prognostic tool for PCa

In terms of PCa, the reports on exosomes are very few. One of the first studies reported no apparent differences between exosomes of benign origin and malignant origin, regarding their synthesis, storage and release.[68] Most likely, these vesicles may differ in biochemical properties. Unfortunately, so far, no high-throughput techniques, such as mass spectrometry and microarray, have been used to evaluate the differences between exosomes of benign origin and malignant origin to identify new biomarkers. Four studies used mass spectrometry to profile exosomes derived from PCa cell lines, xenografts and metastases. To search for PCa-secreted proteins, serum from PCa-xenografted mice was analysed by mass spectrometry. All of the identified proteins were screened for human-specific sequences by extensive database searching. The proteins containing human-specific sequences were of PCa origin. Interestingly, the subcellular localisation of most of these proteins is cytoplasmic, supporting the idea that these proteins are secreted in mouse blood through exosomes. Indeed, proteomic profiling of exosomes derived from human PCa cell lines confirmed the presence of almost all of the previous identified serum proteins.[26] Two other studies analysed vesicles from prostate cell lines and vertebral prostate cancer metastases by mass spectrometry; they identified proteins related to angiogenesis, signal transduction pathways and cancer progression [48,69], including caveolin-1 (Cav-1), Akt, pyruvate kinase M2 (PKM2), programmed cell death 6 interacting protein (PDCD6IP) and poly(A)-binding protein 1 (PABPC1). Subsequent *in vitro* functional assays (such as migration and proliferation assays) demonstrated that these vesicles can influence cancer microenvironment and promote cancer progression. Although these findings are promising, further investigations are needed to fully elucidate the role of PCa exosomes in cancer development.

Aside from these biological studies, exosomes and exosomal content from patient samples have also been evaluated for their potential as potential biomarkers. Urinary exosomes from 10 organ-confined PCa patients undergoing hormonal therapy prior to radical radiotherapy were analysed.[58] Other than a considerable variation in the quantity of total exosomal proteins, no difference was observed between healthy men and PCa patients. Although these results do not specify which proteins are present in exosomes, it emphasises the technical feasibility of assessing exosomal proteins to evaluate the clinical status of PCa. However, better sample preparation, such as immunoaffinity isolation, and more robust technical approaches are needed to define significant differences with such a huge variation.

RNA expression analysis of urine-derived and PCa cell line-derived exosomes revealed that the known RNA-markers for PCa, such as the *TMPRSS2:ERG* fusion gene and *PCA3*, can be detected in exosomes by RT-PCR.[10] The *TMPRSS2:ERG* fusion transcripts were detected in urinary exosomes from two patients with high Gleason scores but not in those from two patients with low Gleason scores.[47] *PCA3* mRNA was detected in exosomes

derived from all patients. Interestingly, none of the hormone-treated patients showed detectable levels of *TMPRSS2:ERG* or *PCA3* RNAs, suggesting that the response to treatment might reduce the size of PCa tissue and, thereby, decrease the expression levels of these androgen-responsive genes.

Exosomes in other urological malignancies

Very few studies on exosomes in other urological malignancies are available. One group (Welton *et al.*) published a report on the profiling of exosomes from a bladder cancer cell line.[70] They measured exosomes derived from a single bladder cancer cell line by mass spectrometry and identified a set of protein biomarkers associating with bladder cancer, such as multiple tetraspanins and alfa-6 integrins . In respect of renal cell carcinoma, Zhang *et al.* evaluated the effects of exosomes as an immunotherapy tool by expressing GPI-IL-12 on exosomal membranes.[71] Implementation of this protein in exosomes significantly promoted T cell proliferation, contributing to an enhanced cytotoxic effect of these T cells. This effect may improve tumor rejection, therefore suggesting that exosomes may have potential application in immunotherapy.

Considerations

The studies on small vesicles in PCa describe the first step in developing new methods and identifying novel markers for the diagnosis and prognosis of PCa. Although the initial results are promising, further investigations are required to assess the exact clinical values and the biological functions of exosomes.

To investigate prostate- or PCa-derived exosomes from complex body fluids, current isolation protocols (such as ultracentrifugation) are not optimal. Procedures according to these protocols result in a heterogeneous sample of exosomes derived from several different organs. Organ-specific isolation can be achieved by immunoaffinity capture beads coated with antibodies directed against organ- or cancer-specific proteins. Experiments using latex or magnetic beads have been successfully used to achieve specific purification.[47]

For biomarker discovery from body fluids, it is important to decide which type of fluid to use. To search for markers from the prostate, serum, urine and semen are the obvious options. Collecting urine is less invasive, compared to drawing blood through venipuncture, and urinary exosomal proteins are generally more stable because the proteolytic activity in urine is lower than that in serum.[6] When urine is collected for prostasome/exosome study, the procedure is preferentially performed after prostate massage to increase the quantity of exosomes.[58]

Evaluation of exosomal content from retrospective samples with different tumor characteristics and a generally long follow-up may provide us novel diagnostic and prognostic biomarkers for PCa. To use retrospective biobank samples, the knowledge

of collection, storage, and processing conditions of urinary and plasma serum samples is essential. Storage without multiple cycles of thawing and freezing of whole urine at -80 degrees Celsius does not seem to affect exosomal content [6]. Exosomes can resist endogenous proteolytic activity in urine for at least 18 hours at 37 degrees Celsius. These findings indicate that exosomes are quite stable in complex body fluids.

CONCLUSIONS

Exosomes are small vesicles (50 to 100 nm) secreted by almost all tissues, representing their tissue origin. By isolating these exosomes, several problems of biomarker discovery from complex body fluids can be largely solved. Therefore, purification of prostate (cancer)-derived exosomes will allow us to profile the exosomes, providing a promising source of protein and RNA biomarkers for PCa. This profiling will contribute to the discovery of novel markers for the early diagnosis and reliable prognosis of PCa. Although the initial results are promising, further investigations are required to assess the clinical value of these exosomes in PCa.

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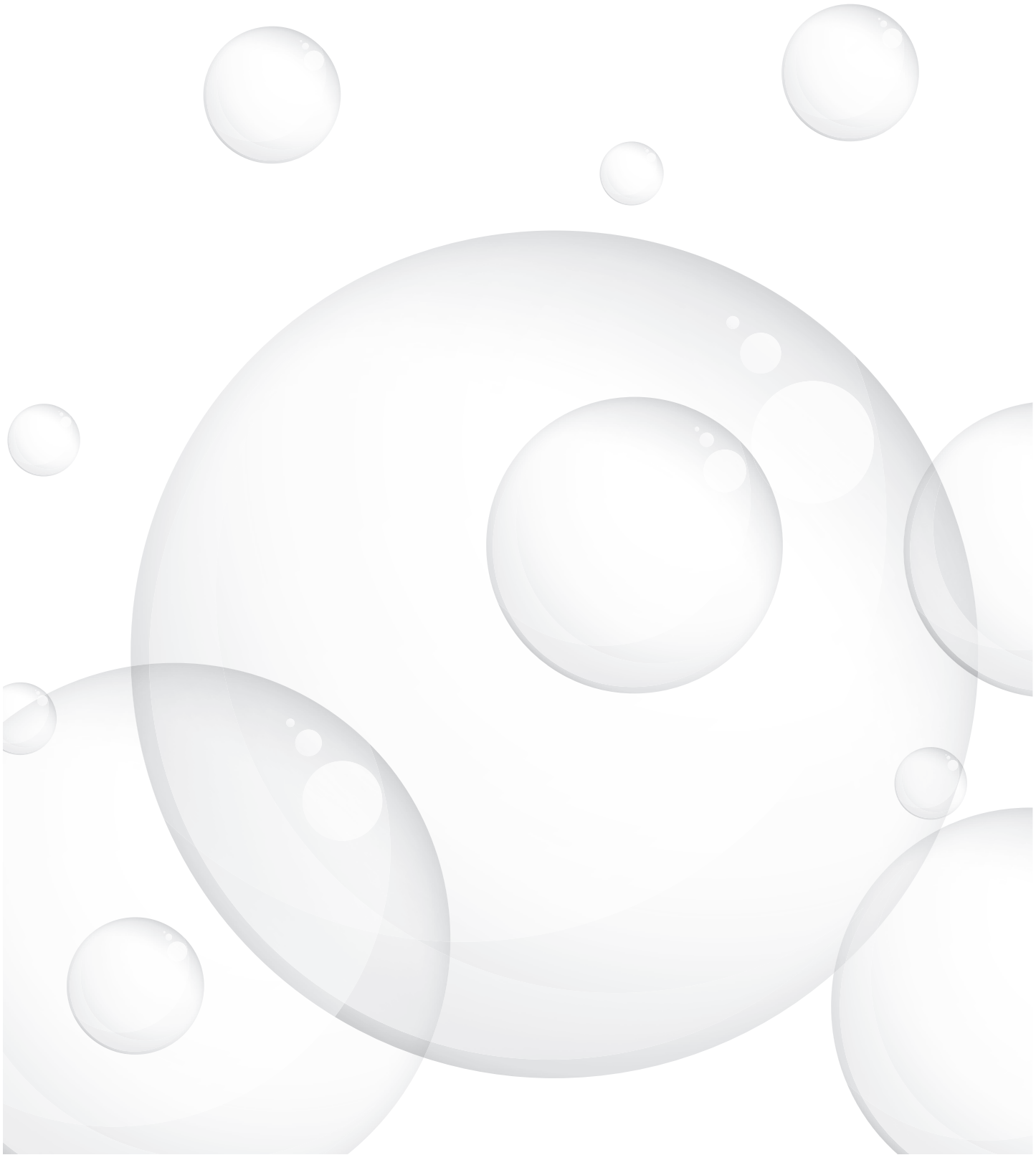

4

Proteomic profiling of exosomes leads to the identification of novel biomarkers for prostate cancer

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ABSTRACT

Background

Current markers for prostate cancer, such as PSA lack specificity. Therefore, novel biomarkers are needed. Unfortunately, the complexity of body fluids often hampers biomarker discovery. An attractive alternative approach is the isolation of small vesicles, i.e. exosomes, ~100 nm, which contain proteins that are specific to the tissue from which they are derived and therefore can be considered as treasure chests for disease-specific biomarker discovery.

Materials and Methods

Exosomes were isolated from 2 immortalized primary prostate epithelial cells (PNT2C2 and RWPE-1) and 2 PCa cell lines (PC346C and VCaP) by ultracentrifugation. After tryptic digestion, proteomic analyses utilized a nanoLC coupled with an LTQ-Orbitrap operated in tandem MS (MS/MS) mode. Accurate Mass and Time (AMT) tag approach was employed for peptide identification and quantitation. Candidate biomarkers were validated by Western blotting and Immunohistochemistry.

Results

Proteomic characterization resulted in the identification of 248, 233, 169, and 216 proteins by at least 2 peptides in exosomes from PNT2C2, RWPE-1, PC346C, and VCaP, respectively. Statistical analyses revealed 52 proteins differently abundant between PCa and control cells, 9 of which were more abundant in PCa. Validation by Western blotting confirmed a higher abundance of FASN, XPO1 and PDCD6IP (ALIX) in PCa exosomes.

Conclusions

Identification of exosomal proteins using high performance LC-FTMS resulted in the discovery of PDCD6IP, FASN, XPO1 and ENO1 as new candidate biomarkers for prostate cancer.

INTRODUCTION

Prostate Specific Antigen (PSA) is a clinically useful protein biomarker for diagnostics and follow-up after treatment for prostate cancer (PCa). Nevertheless, PSA-based screening was shown to have a high risk of overdiagnosis and overtreatment because it lacks specificity.[1,2] In order to differentiate more accurately between benign prostate diseases and (different forms) of PCa, prevent unnecessary prostate biopsies, and support the urologist in recommending optimal treatment, new molecular biomarkers are urgently needed.

In the past few decades, a tremendous amount of research has been performed to find new and better biomarkers for PCa, often using state-of-the-art mass spectrometry technologies, but the discovery of novel low abundance protein has been generally hampered by the complexity of serum or urine.[3] Isolation of exosomes from body fluids represents an attractive approach to bypass these limitations and enable detection of candidate (low abundant) biomarkers.

Recent findings in the search for new biomarkers have revealed that small exosomes (50-150 nm), are present in serum and urine.[4] By isolating exosomes from body fluids it should be possible to overcome the dynamic range challenge and facilitate characterization of tissue/cancer-derived proteins that might more accurately represent cellular conditions. Therefore exosomes could be useful for determining individual tumor characteristics.[5]

In this study, our goal was to determine the presence and significance of exosomal proteins as novel candidate biomarkers for PCa by comparing exosomes from non-cancerous prostate cell lines to exosomes from PCa cell lines.

MATERIAL AND METHODS

Cell culture and isolation

Two human immortalized prostate epithelial cell lines (PNT2C2[6] and RWPE-1) and two PCa cell lines (PC346C[7] and VCaP[8]) were cultured in 10 T175 (175 cm^2) culture flasks (Greiner Bio-One, Frickenhausen, Germany) up to 80-100% confluency. The PNT2C2 and VCaP cell line were cultured in RPMI 1640 (Lonza, Verviers, Belgium) and supplemented with 5% and 10% FCS, 500 U penicillin and 500 U streptomycin (Lonza, Verviers, Belgium). The RWPE-1 cell line (ATCC-LGR, Wesel, Germany) was cultured in Keratinocyte Serum Free Medium (Invitrogen, CA, USA) and supplemented with 5 ml Pen-Strep and a commercial kit containing Bovine Pituitary Extract (BPE, 0.05 mg/ml) and Epidermal Growth Factor (EGF, 5 ng/ml). The PC346C cell line was cultured in Dulbecco's modified Eagle's medium-Ham's F-12 medium (Lonza), supplemented with multiple additives as described by Marques.[9]

After reaching 80-100% confluency, the cells were incubated with 25 ml serum free medium. After 48 h, the supernatant was collected and subjected to centrifugation steps of 400 x*g* (10 min), 3000 x*g* (20 min), and 10,000 x*g* (30 min) to remove cellular debris. Exosomes were then pelleted at 64,000 *g* (110 min), and at 100,000 *g* (Sucrose gradient) for 1 h.[10] At least two separate exosomes isolations from each of the four cell lines were pooled. Total amount and concentration of exosomal proteins of the pooled samples was measured with a BCA-assay (Pierce, Rockford, IL, USA).

Electron Microscopy (EM)

5 µL of exosomes were spotted onto Formvar-coated grids (200 mesh) and fixed in 2% paraformaldehyde. After fixation the exosomes were negatively stained using 4% uranylacetate. Grids were examined by a Philips CM100 electron microscope at 80 kV.

Sample preparation for Mass Spectrometry

TFE (2,2,2-Trifluoroethanol) (Sigma-Aldrich) was added to the samples to a final concentration of 50%. The samples were sonicated in an ice-water bath (Branson 1510, Danbury, CT) for 2 minutes and then incubated at 60 ^{0}C for 2 h with constant shaking (300 rpm). For protein disulfide bridge (S-S) reduction, DTT (Dithiothreitol) (Sigma-Aldrich) was added at final concentration of 2 mM, followed by sonication for 2 min. The samples were spun down and incubated at 37 ^{o}C for 1 h with shaking (300 rpm). The samples were diluted, 5-fold with 50 mM ammonium bicarbonate (pH 7.8) prior to adding sequencing grade modified trypsin (Promega, Madison, WI) for protein digestion (1:50 w/w trypsin-to-protein). The samples were shaken (300 rpm) over-night (16 h). Rapid freezing of the samples in liquid nitrogen quenched the digestion. All samples were concentrated down in Speed-Vac SC 250 Express (Thermo Savant, Holbrook, NY).

Mass spectrometry

Proteomic measurements were performed using a nanoLC-MS at the Environmental Molecular Science Laboratory (EMSL), Richland, WA, USA. The analytical platform consisted of an on-line constant pressure (5000 psi) reversed-phase (C^{18}) liquid chromatography (RPLC) system [150 µm i.d. × 360 µm o.d. × 65 cm capillary (Polymicro Technologies Inc., Phoenix, AZ)] coupled to an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA) via an electrospray ionization (ESI) source manufactured in-house.[11] Briefly, full MS were acquired over *m/z* range of 400-2000 at resolution of 100,000, followed by data-dependent LTQ MS/MS for the top six most abundant ions in each full MS scan, using a collision energy setting of 35% and dynamic exclusion time of 60 s. An exponential HPLC gradient of ~100 min (from 0 – 70% B) was used for each analysis, with mobile phases consisting of 0.1% formic acid in water (A) and 0.1% formic acid in ACN (B). Each

sample was analyzed in triplicate, with approximately 5 µg of total peptide consumed (i.e., loaded on the column) in each analysis.

Mass Spectrometry Data Analysis

The resulting MS data was analyzed using the PNNL developed Accurate Mass and Time (AMT) Tag pipeline.[12] SEQUEST software[13] was used to search tandem mass spectra against the UniProt human database (download on April 5 2011). Confidently identified peptides (Suppl. Table 1) were assembled into an exosome-specific AMT tag database. For comparative analyses, LC-MS features were matched against AMT tags for identification and relative MS-peak intensities were used to derive change in abundance. AMT tag approach facilitated quantitation of many more peptides than spectral counting alone. As long as a peptide was identified in at least one sample/analysis (by tandem MS), it could be quantified in all datasets where it was detected, even if the LC-MS feature was not abundant enough to be fragmented in that particular analysis. VIPER software[14] was used to correlate AMT tag entries (identified peptides) with LC-MS features relying on high mass measurement accuracy (MMA <2 ppm) and normalized elution time accuracy (NET ~2.5%). Consequently, each LC-MS feature matched back to a single peptide (AMT tag) thereby giving a peak intensity value (or relative abundance) for that peptide. For redundant peptide identifications in the case of a single peptide matching multiple proteins (typically protein isoforms) a representive protein was chosen; therefore, each reported peptide matches back to a single protein. No peptide identifications were made on mass alone.

For the 263 identified proteins, the Human Protein Reference Database (HPRD) and Ingenuity Pathway Analysis (IPA) were used to determine subcellular location and biological function.[15]

Selection of potential protein biomarkers for prostate cancer was performed using two independent approaches. First, proteins were selected that were present in both PCa cell line derived exosomes and absent in both non-PCa exosomes. With the second approach, DAnTE software[16] was used to convert peptide peak intensity values to a log2 scale and assess them at a protein level using Rrollup (reference peptide based scaling) parameters where peptides were excluded from scaling if they were not seen in at least three datasets and no minimum peptide presence was required. Proteins presented in this manuscript were identified by at least two peptides. ANOVA pairwise comparisons between each PCa and control cell line were also performed in DAnTE where the minimum number of data points per factor level was set at three, so that in order for a protein to show statistically significant changes it would have to be identified in all three replicates. Significant difference was determined as a p-value and q-value lower than 0.05. DAnTE generates p-values and estimates their q-values. The q-value of a test measures the proportion of false positives incurred (called the false discovery rate) when that par-

ticular test is called significant. Only the significantly different proteins were selected for unsupervised hierarchical clustering. TreeView software was used to log transform and mean center expression values, and subsequently cluster all the proteins based on their expression. To further select the most promising proteins, a ≥1.5 log2 fold change cutoff was applied along with a requirement that each protein showed significant change in at least two of the four comparisons listed in Table 1. Table 1 lists the resulting 52 proteins, 9 of which showed increased (and 43 decreased) abundance in exosomes derived from the PCa cells.

To further select the most promising proteins from the two approaches, proteins were scaled based on prostate preferentiality. Five different human gene expression atlases[17-21] based on microarray expression data were combined in SRS[22], to determine protein-corresponding gene expression. Eventually, prostate preferentiality was determined as 1.5 fold higher expression in prostate tissue compared to kidney and bladder tissue using gene expression microarray data.[23]

Table 1. Proteins with significant abundance changes (>1.50 log2 fold) between prostate cancer and immortalized primary prostate epithelial cell lines.

Protein Description (UniProt Accession #)	gene_ symbol	PC346C/ PNT2C2	PC346C/ RWPE	VCaP/ PNT2C2	VCaP/ RWPE
Programmed cell death 6-interacting protein(Q8WUM4)*	PDCD6IP	1.64	3.28	1.95	3.59
Elongation factor 1-alpha 2(Q05639)	EEF1A2	1.92	3.18		1.83
Fatty acid synthase(P49327)*	FASN	1.67	4.06		2.52
Ubiquitin-60S ribosomal protein L40(P62987)	UBA52		2.44	1.98	3.03
Vacuolar protein sorting-associated protein 28 homolog(Q9UK41)	VPS28	2.22	3.14		2.13
Actin-related protein 3B(Q9P1U1)	ACTR3B		5.71		5.27
Basal cell adhesion molecule(P50895)	BCAM			1.95	1.95
CD9 antigen(P21926)*	CD9		4.13		2.58
Polyadenylate-binding protein 1(P11940)	PABPC1	2.89		3.24	
14-3-3 protein beta/alpha(P31946)	YWHAB	-4.88	-4.11	-2.42	-1.64
Annexin A2(P07355)	ANXA2	-7.86	-5.10	-5.60	-2.84
Sodium/potassium-transporting ATPase subunit alpha-1(P05023)	ATP1A1	-3.23	-2.87	-3.38	-3.01
Sodium/potassium-transporting ATPase subunit beta-1(P05026)	ATP1B1	-3.68	-3.03	-3.52	-2.87
Sodium/potassium-transporting ATPase subunit beta-3(P54709)	ATP1B3	-2.79	-2.04	-2.33	-1.58
Basigin(P35613)	BSG	-2.90	-3.56	-4.62	-5.28
Chloride intracellular channel protein 1(O00299)	CLIC1	-5.03	-2.85	-4.34	-2.16
Integrin alpha-6(P23229)	ITGA6	-2.34	-4.84	-2.21	-4.72

Table 1. Proteins with significant abundance changes (>1.50 log2 fold) between prostate cancer and immortalized primary prostate epithelial cell lines. (continued)

Protein Description (UniProt Accession #)	gene_ symbol	PC346C/ PNT2C2	PC346C/ RWPE	VCaP/ PNT2C2	VCaP/ RWPE
Junctional adhesion molecule A(Q9Y624)	F11R	-1.56	-1.58	-2.15	-2.17
Actin, aortic smooth muscle(P62736)	ACTA2	-3.61		-3.97	-1.83
Potassium-transporting ATPase alpha chain 2(P54707)	ATP12A	-3.67	-2.32	-2.89	
Catenin beta-1(P35222)	CTNNB1	-5.02	-1.73	-2.90	
Alpha-enolase(P06733)*	ENO1	-3.63	-1.82	-2.18	
78 kDa glucose-regulated protein(P11021)	HSPA5	-3.86		-3.56	-2.08
Importin subunit beta-1(Q14974)	KPNB1	-5.13	-2.02	-2.15	
Pyruvate kinase isozymes M1/M2(P14618)	PKM2	-4.02	-2.88	-2.19	
Triosephosphate isomerase(P60174)	TPI1	-3.67	-2.19	-1.62	
14-3-3 protein epsilon(P62258)	YWHAE	-3.70	-2.73		
14-3-3 protein theta(P27348)	YWHAQ		-3.17		-1.85
4F2 cell-surface antigen heavy chain(P08195)	SLC3A2	-4.01	-5.90		
ADP-ribosylation factor 1(P84077)	ARF1	-3.81		-3.12	
CD151 antigen(P48509)	CD151			-4.41	-3.12
Coxsackievirus and adenovirus receptor(P78310)	CXADR	-2.70		-1.96	
EH domain-containing protein 4(Q9H223)	EHD4	-2.77	-2.18		
Prostaglandin F2 receptor negative regulator(Q9P2B2)	PTGFRN		-1.91		-2.76
Putative heat shock protein HSP 90-beta 2(Q58FF8)	HSP90AB2P	-3.60	-1.87		
Putative heat shock protein HSP 90-beta-3(Q58FF7)	HSP90AB3P	-4.05		-2.35	
Hemoglobin subunit beta(P68871)	HBB		-5.21		-5.19
Ras GTPase-activating-like protein IQGAP1(P46940)	IQGAP1	-5.14		-4.40	
Keratin, type I cytoskeletal 9(P35527)	KRT9	-1.54	-1.88		
Keratin, type II cytoskeletal 2 epidermal(P35908)	KRT2	-1.64	-1.92		
Lactadherin(Q08431)	MFGE8	-2.02	-2.19		
Protein DJ-1(Q99497)	PARK7	-1.80	-2.37		
Phosphoglycerate kinase 1(P00558)	PGK1	-2.28		-2.13	
Peroxiredoxin-1(Q06830)	PRDX1	-2.43		-2.14	
Ras-related protein Rab-10(P61026)	RAB10	-3.10		-3.11	
Ras-related protein Rab-1A(P62820)	RAB1A	-2.93	-2.11		
Ras-related C3 botulinum toxin substrate 1(P63000)	RAC1		-1.63		-2.42
Ras-related protein Rap-1A(P62834)	RAP1A	-3.01		-2.95	
Adenosylhomocysteinase(P23526)	AHCY	-1.97	-1.68		
Tubulin alpha-1A chain(Q71U36)	TUBA1A	-3.42		-1.80	
T-complex protein 1 subunit epsilon(P48643)	CCT5	-2.72	-2.85		
UDP-glucose 6-dehydrogenase(O60701)	UGDH	-3.58		-3.13	

Western blotting

From every exosome sample 5 µg of protein was mixed with Laemmli sample buffer (1:1), heated at 95^{0}C for two minutes and loaded onto 10% one-dimensional SDS-PAGE gels. Subsequently, proteins were transferred onto Protran nitrocellulose membranes (Whatman's Hertogenbosch, the Netherlands) and blocked (1h) at room temperature with 5% nonfat dry milk in Tris-Buffered Saline with 0.1% Tween-20. Then, the gels were incubated overnight at 4^{0}C with antibodies against: PDCD6IP (1:500 dilution, Sigma-Aldrich), FASN (1:500 dilution, Sigma-Aldrich), XPO1 (1:200 dilution, Santa Cruz Biotechnology, Heidelberg, Germany), ENO1 (Clone H300, 1:1000 dilution, Santa Cruz Biotechnology), GAPDH (Clone 7B, 1:500 dilution, Santa Cruz Biotechnology), CD9 (Clone 209306, 1:500 dilution, R&D Systems, Abingdon, UK), PSA (Clone A0562, 1:500 dilution, DakoCytomation, Heverlee, Belgium). Secondary antibodies (HRP-conjugated Goat anti Mouse/Rabbit, 1:10,000 dilutions, DakoCytomation) were incubated for 1 h. BM Chemiluminescence Blotting Substrate (POD, Roche Applied Science, Almere) was used to initiate the oxidation by HRP.

Immunohistochemistry (IHC)

IHC expression analysis of candidate biomarkers was performed on: normal prostate tissue (NAP, n=2), PCa Gleason score 3+3=6 (n=2), and PCa Gleason score 5+4=9 (n=2). Tissues slides were mounted on aminoacetylsilane coated glass slides (Starfrost, Berlin, Germany), deparaffinised in xylene and dehydrated in ethanol. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in PBS for 20 min. Microwave pretreatment was performed for 15 min in tris(hydroxymethyl)aminomethane-EDTA (pH 9.0). After pretreatment, the slides were incubated with the PDCD6IP (1:400), FASN (1:50), and XPO1 (1:50) antibodies, overnight at 4 ^{0}C. Subsequently, the EnVision DAKO kit (DAKO, Glostrup, Denmark) was used for chromogenic visualization. After staining the slides were counterstained with hematoxylin, washed, dehydrated and mounted in malinol (Chroma-Geselschaft, Körgen, Germany).

RESULTS

Isolation and characterization

Electron Microscopy (EM) of the purified exosome samples revealed that vesicles derived from four cell lines are reasonably homogeneous in size, with an approximate diameter of 70-200 nm (Figure 1). LC-MS/MS analyses after tryptic digestion, identified 1494 non-redundant peptides (Suppl. Table 1), corresponding to 496 proteins by at least 1 peptide (Suppl. Table 2). 263 proteins were identified by at least 2 peptides, and specifically 248, 233, 169, and 216 proteins were identified in the PNT2C2, RWPE-1, PC346C and VCaP

cell lines, respectively (Suppl. Table 3). Unsupervised hierarchical clustering of these 263 proteins resulted in a clear distinction between cancer and control cell lines (Figure 2).

The identified exosomal proteins in the 4 cell lines showed similar subcellular localization patterns (Figure 3A). When compared to all proteins included in the IPA database, exosomes contain, relatively speaking, more cytoplasmic proteins and almost no extracellular proteins. A majority of proteins detected within exosomes relate to tumorigenesis, cell death, protein synthesis, cellular growth and proliferation (Figure 3B).

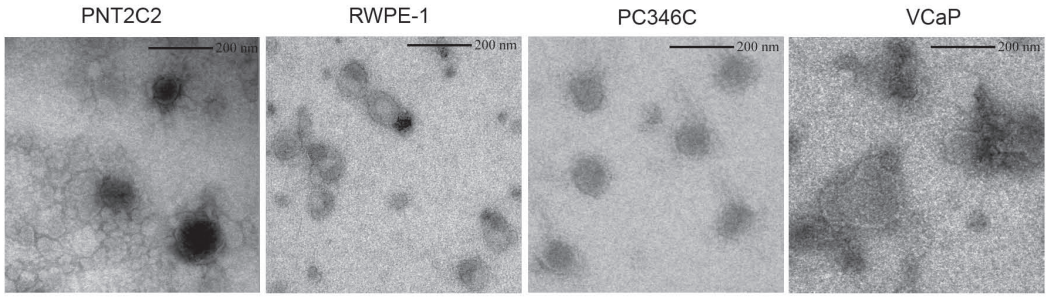

Figure 1. Electron microscopic (EM) images of purified exosomes derived from the PNT2C2, RWPE-1, PC346C and VCaP cell lines. All exosome samples contain multiple vesicles with a size in the range of 70-200 nm. The darkness of the vesicles reflects the difference in density of exosomes between samples.

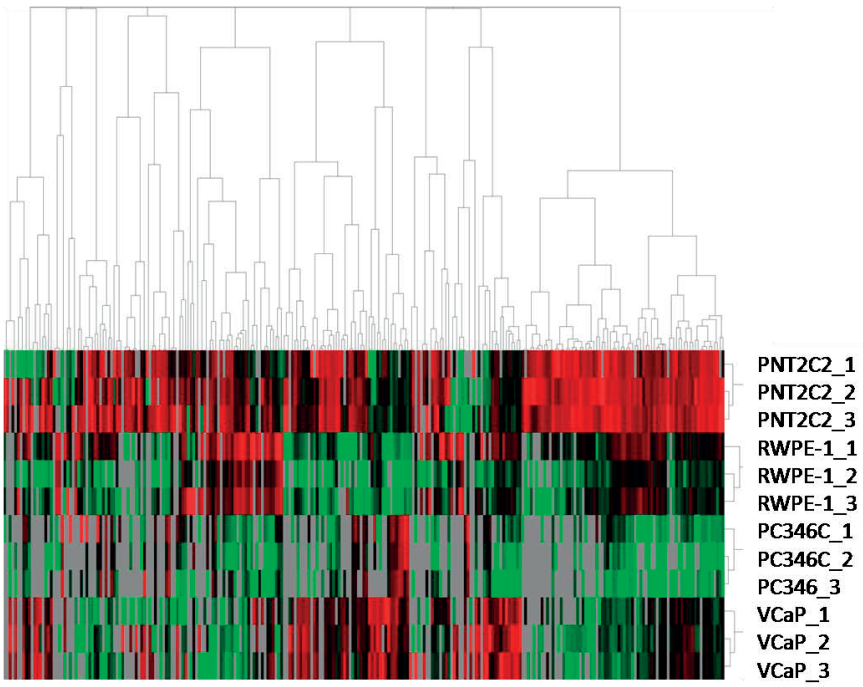

Figure 2. Unsupervised hierarchical clustering of differentially abundant proteins (n=263 proteins with >2 peptides) based on their MS-peak intensity values. Each exosome sample was analyzed in triplicate. Results were mean centered and log-transformed. Relative protein abundance is colored-coded with red corresponding to a relatively high abundance, green r corresponding to a relatively low abundance, and grey indicating missing abundance values.

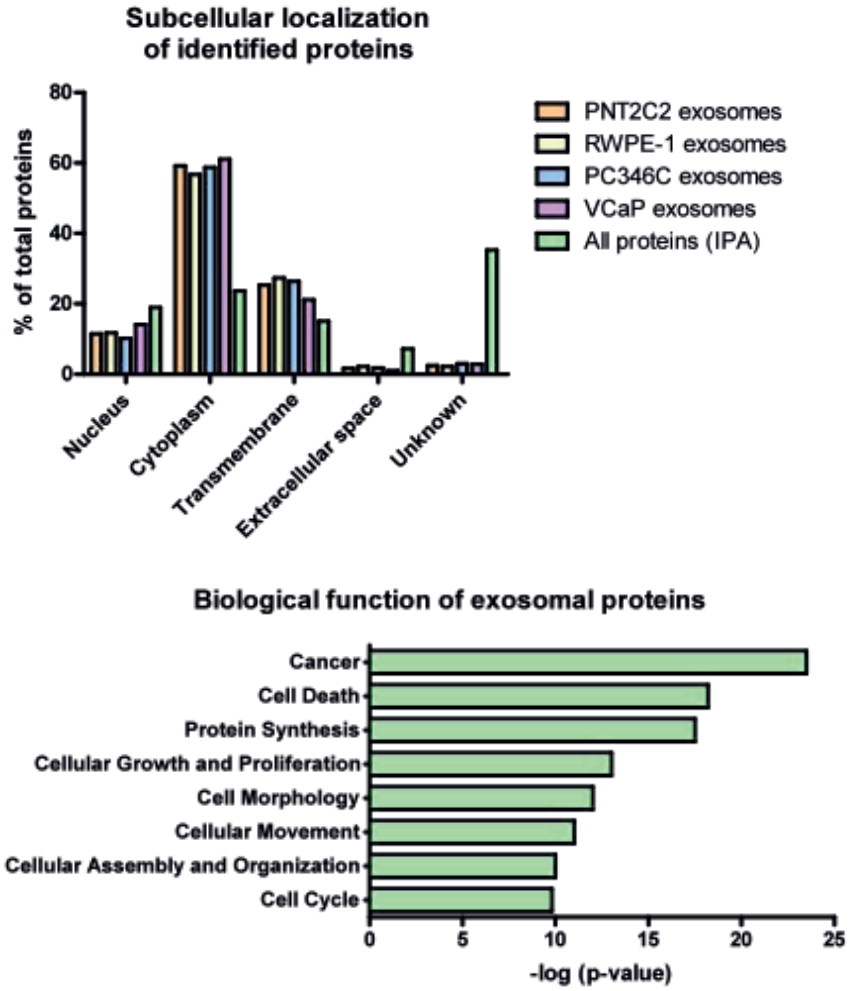

Figure 3. A. Subcellular assignment of the proteins identified within the different samples. Exosomes from all four cell lines (PNT2C2, RWPE-1, PC346C, VCaP) contained 60% of cytoplasmic proteins and 25% of transmembrane proteins. B. The top seven functions of exosomal proteins according to Ingenuity Pathway Analysis. Fisher's exact test was applied to calculate significance (p-value < 0.05).

Selection of potential biomarkers

To select proteins that show significant change in abundance between the PCa exosomes and non-PCa exosomes we used ANOVA pairwise comparisons (i.e., p-value and q-value <0.05, presence in all analyses, ≥ 2 peptides).[16] Supplemental Tables 5-8 contain results obtained for the PC346C (PCa) vs. PNT2C2 (control), PC346C (PCa) vs. RWPE-1 (control), VCaP (PCa) vs. PNT2C2 (control), and VCaP (PCa) vs. RWPE-1 (control). To further improve confidence, we required that each protein was determined to be significantly changing in abundance in at least 2 comparisons; this further reduced our list to 52 proteins (Table 1).

Our proteomic analysis indicated PDCD6IP, FASN, CD9, and ENO1 to have significant change in abundance between two conditions, while XPO1 did not pass our stringent filtering criteria and was therefore considered unchanged in abundance in the VCaP vs. RWPE-1 comparison (Suppl. Table 8). Even so, we chose to validate XPO1 because of its higher abundance in VCaP exosomes compared to the RWPE-1 control and the availability of a high quality antibody suitable for Western blotting and immunohistochemistry.

Exploration of novel candidate biomarkers

For FASN and XPO1, strong signals were observed in whole cell lysates as compared to the exosomes and there appears to be relatively higher abundance within the VCaP exosome sample (Figure 4). The protein PDCD6IP is enriched in exosomes and shows higher abundance in both PCa-derived exosome samples as depicted in Figure 4 and Table 1. Based on the MS analyses, FASN is significantly higher in the PC346C exosomes compared to both controls and in VCaP exosomes compared to RWPE-1 control. This higher abundance of FASN in PC346C is confirmed by the Western blot. CD9 is highly enriched in exosomes and shows relatively high abundance in the PC346C exosomes. XPO1 exhibited higher abundance in the VCaP exosomes compared to controls. MS data characterized ENO1 to be significantly decreased in abundance in PC346C compared to both controls and in VCaP compared to the PNT2C2 control. Western blotting of ENO1 revealed an additional band (approximately 30 kDa) within the two PCa-derived exosome samples. As expected, based on the difference in PSA-secretion and exosome formation, PSA is predominately present in the two cancer cell samples and absent in exosomes. Supernatants that were collected after exosomes were pelleted during ultracentrifugation, did not contain any of the exosomal proteins, except ENO1 and GAPDH uniquely in VCaP medium.

Validation on clinical samples

PDCD6IP showed strong luminal and basal epithelial cytoplasmic staining in normal adjacent prostate (NAP), with no alteration in protein expression in PCa tissue with different Gleason scores (Figure 5). In NAP, FASN is moderately to highly abundant in epithelial cells. Nevertheless, when Gleason scores increases, staining becomes stronger. Regarding XPO1, there is a strong nuclear abundance in NAP and a weak cytoplasmic staining. Within PCa cells, the cytoplasmic abundance increases with Gleason scores. Nuclear staining remains equal among all PCa tissues.

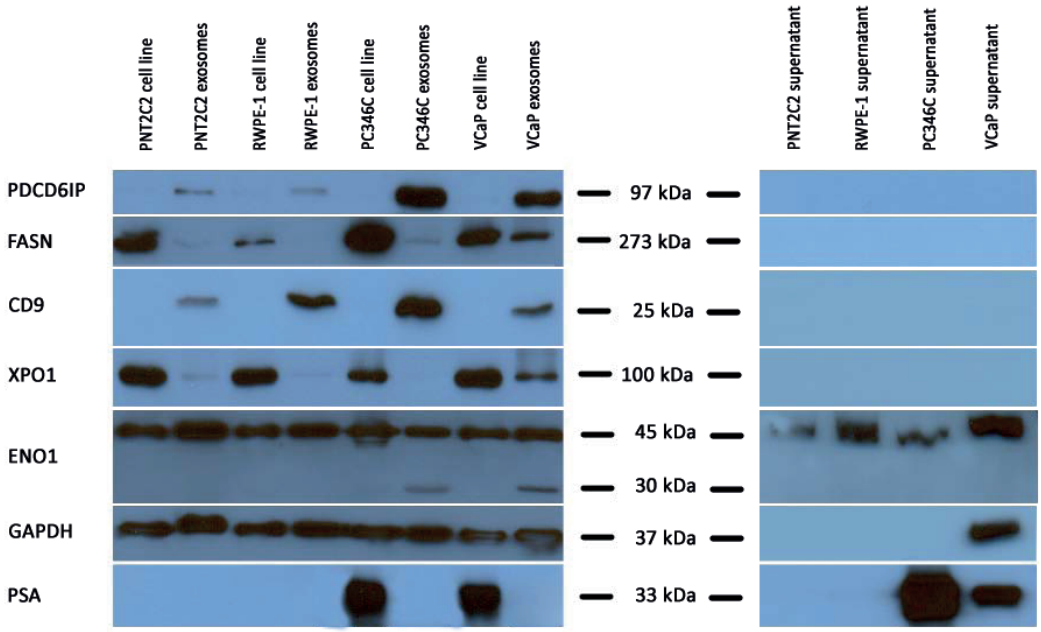

Figure 4. Validation of protein expression by Western blotting. All four exosome samples and their corresponding cell lines were used for validation. Furthermore, supernatant from the pelleted exosomes was used as a control. The selected proteins FASN, XPO1, CD9 and PDCD6IP, were tested with ENO1 and GAPDH as controls. PSA was tested to confirm it is secreted through alternative secretion pathway and therefore not present within exosomes. The nearest protein marker (kDa) is indicated for each blot.

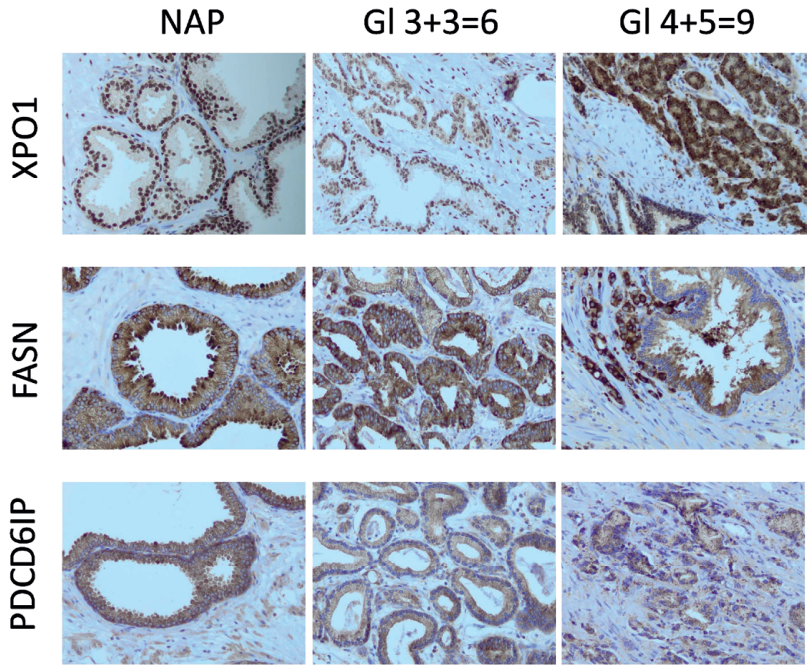

Figure 5. XPO1, FASN and PDCD6IP abundance by immunohistochemistry on normal adjacent prostate (NAP), low-grade prostate cancer (Gleason score 3+3=6) and high grade prostate cancer (Gleason score 4+5=9). Representative pictures of the staining from 2 independent samples per group.

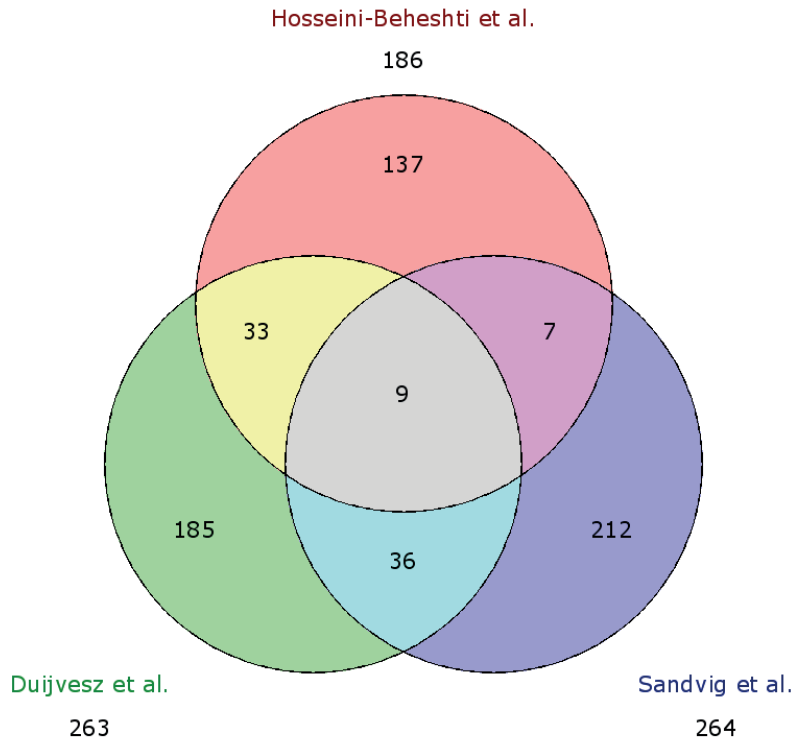

Figure 6. Comparison of proteins identified by Hosseini-Beheshti *et al*., Sandvig *et al*. and this manuscript visualized by a Venn diagram. Every area represents the logical relations all identified proteins.

DISCUSSION

Comparison of exosomes derived from cancerous and non-cancerous cell lines provides a powerful tool to overcome the dynamic range challenge and identify novel low abundant cancer-derived biomarkers. This unique approach within exosomal protein research, combined with state-of-the-art LC-MS analyses facilitated identification of novel candidate biomarkers for PCa. So far, this manuscript is the first that describes expression amongst multiple PCa cell lines, but also analyses exosomes from multiple non-malignant prostate epithelial cell lines. This comparison enables us to identify PCa specific candidate biomarkers more properly. Also this paper is the first that describes validation of the expression of candidate protein markers on clinical patient samples, implying their true value as biomarkers.

The total number of unique proteins we identified in this study (496 by ≥1 peptide, 263 by ≥2 peptides), is comparable with previously published exosome proteomic reports.[24-28]. Assignment of a subcellular localization revealed that a large proportion of exosomal proteins normally locate in the cytoplasm or nucleus of cells. After comparing this to a database containing a vast majority of all proteins (~20,000), we noticed that exosomes have a relatively comparable abundance of nuclear proteins, higher

abundance of cytoplasmic proteins and a substantially lower abundance of extracellular proteins. This fits the current theory of exosome formation.[4] Exosomes display an over-representation of transmembrane and cytoplasmic proteins, such as CD9 and PDCD6IP, as shown by Western blots. This finding agrees with the theory that biogenesis and selection of exosomal content is not a random procedure, but at least partly the result of a selective sorting process.[4]

Two recent proteomic studies revealed exosomal proteins related to prostate cancer;[28,29] Sandvig et al. performed LC-MS analysis on a single prostate (cancer) cell line, were Hosseini-Beheshti examined five PCa cell lines and one non-malignant prostate epithelial cell line. They both reported 266 and 220 proteins, which is similar to the number we revealed. Interestingly, Sandvig et al. reported a different protein subcellular distribution and correlation with biological processes as compared to our data. Sandvig et al. proposed CDCP1 and CD151 as candidate markers, were Hosseini-Beheshti suggested ANXA2, CLSTN1, FLNC, FOLH1 and GDF15. Hosseini-Beheshti et al. also showed FASN to be an exosomes-derived candidate biomarker (in agreement with our results). When we compare their identified proteins (by >2 peptides) we noticed an overlap of only 9 proteins, respectively CD9, ANXA1. ACTB, PGK1, RAN, EPCAM, HSPB1, PDCD6IP and PRDX1 (Figure 6).

These proteins have been published previously in multiple articles and are considered to be present in almost all exosomes. PDCD6IP has also been identified by both researchers, but has not been found to be marker for PCa. A total of 199 proteins have uniquely been found in our dataset, which contains our proposed and most promising candidate biomarker XPO1. The relative majority of overlap is with Hosseini-Beheshti et al (42 proteins in total), most probably because we analyzed exosomes from similar cell lines. Interestingly our data also has an overlap with Sandvig et al (45 proteins), but the overlap between them and Hosseini-Beheshti et al is relatively less. The large difference of proteins identified between all groups could be explained by different mass spectrometry techniques and data-analysis approaches.

We identified PDCD6IP as being enriched in exosomes, especially in PCa exosomes. PDCD6IP, also known as ALIX, is a cytoplasmic protein that is known for its role in apoptosis and is shown to be involved in the pathway of selected sorting by ESCRT-complexes.[30] PDCD6IP has been used as a general marker to prove the presence of exosomes.[31] However, no association was made with a higher abundance in cancer-derived exosomes. A possible explanation for high PDCD6IP abundance in PCa-derived exosomes could be that PCa cells have an altered production of exosomes, where they are unable to regulate the sorting of exosomal content properly anymore. It is also possible that cancerous cells attempt to remove the PDCD6IP protein by exosome secretion to (partially) suppress apoptosis. To complement this theory, other non-PCa related studies have shown that overexpression of PDCD6IP correlates with cell death.[32] Using

IHC, we did not find any difference in PDCD6IP abundance between normal prostate epithelium and PCa tissue.

Both FASN and XPO1 have a higher abundance in PCa exosomes derived from VCaP cells. FASN catalyzes the formation of long chain acids from acetyl-CoA, malonyl-CoA and NADPH and has already been suggested as a marker for PCa.[33,34] Recent studies showed that FASN is primarily expressed in hormone-sensitive cells, promote cell proliferation and that the inhibition of FASN effectively and selectively kills cancer cells.[33] However, these studies were all performed *in vitro*. The VCaP cell line used herein is hormone-sensitive, which could explain the higher abundance of FASN in VCaP-derived exosomes. Cancer cells produce more FASN, likely because it promotes cell proliferation, which could lead to higher incorporation into the exosomes. In agreement with previous results,[35] we also observed a significantly increased abundance in PCa as compared to NAP.

XPO1 has been suggested as a prognostic marker for other types of cancer.[36] XPO1 is a nuclear protein known to be involved in nuclear-cytoplasmic export of signal-bearing (NES) proteins, which play a role in relevant tumor signaling pathways, such as P53, AKT1, HDAC5, the androgen receptor (AR) and the EGFR.[37-39] Our findings indicate that XPO1 could be a potential biomarker for PCa. When this protein is validated on whole section PCa samples with IHC, we observe a strong nuclear expression and a very weak cytoplasmic expression. Interestingly, within cancer cells, this protein seems to translocate into the cytoplasm. When Gleason score increases, cytoplasmic XPO1 expression increases. Why this process occurs remains unclear. In a normal cell, XPO1 has to be transported from the cytoplasm back in the nucleus in order to function as a chaperone protein. If this relocation process is inhibited in cancer, cytoplasmic XPO1 will accumulate and more XPO1 might get incorporated in exosomes.

As published previously, an additional protein band (approximately 30 kDa) appears with Western blotting when using an antibody directed against ENO1 in the PC346C cell line.[40] Here we show that this band is also present in VCaP exosomes and absent in exosomes from two non-PCa cell lines. The origin of the additional band could be a non-specific antibody cross-reaction to another protein, an alternative spliced ENO1, a translated fragment or a breakdown product from the original protein. A known protein isoform called MBP-1 (c-myc promoter-binding protein-1) is produced form the ENO1 gene.[41] MBP-1 is identical in sequence to ENO1 but lacks the first 93 or 96 amino acids. With a calculated molecular mass of 36 kDa, MBP-1 is unlikely the estimated 30 kDa additional band. The observation that this additional band occurs only in both cancerous samples could indicate that it might have a relation to PCa.

Unfortunately, all previous published studies[28,29] were discovery-based, and no correlation was made to clinical samples. This report is the first that shows validation of exosomal proteins as prostate cancer biomarkers on patient samples. A recent report

by Principe et al showed the first identification of more than 900 proteins in exosomes derived from human prostate secretion from patients with low grade PCa and healthy men.[42] When we compared their unique protein expression (presence of >2 peptides) only 31 proteins overlap, from which are multiple annexins (ANXA1-7), peroxiredoxins (PRDX1-6), PDCD6IP and general transmembrane proteins (CD9/CD242/CD44). All these proteins are thought to be present in almost all exosomes.

The new markers we identified came from cell line derived exosomes. It would be interesting to investigate whether these proteins could be identified in exosomes derived from urine samples[43] or even tissue samples.[44] As we showed, some candidate biomarkers could harbor clinical potential value for PCa and therefore should be tested on large cohorts of patient samples to fully elucidate their role as a biomarker for this disease. An option for validation could be a tissue micro-array, containing large numbers of prostate (cancer) tissue. Unfortunately, this assay does not assess exosomal proteins anymore but looks at cellular protein expression. In order to test exosomal proteins on large cohorts of patient samples it would be interesting to develop an assay which could measure exosomal proteins directly from urine of tissue samples. An ELISA-assay could be an option for this comprehensive task, where you can capture prostate-derived exosomes very easily from urine and subsequently perform protein detection assays. The ELISA-assay can be performed in two ways. The first is by using a detection antibody directed against exosomal transmembrane proteins. This technique enables us to identify these transmembrane proteins but also estimate the number of exosomes. The second potential use for this technique is capturing exosomes in the assay, disrupt the membrane by using a detergent and subsequently measure the released intra-exosomal proteins. So far the number of reports on exosome-based ELISA-assays is minimal. More research has to be performed to establish such an ELISA-assay which enables us to measure exosomal proteins.

CONCLUSION

Prostate (cancer) cells secrete exosomes that can be used to identify novel candidate biomarkers for PCa. Identification of exosomal proteins by high performance LC-FTMS resulted in the discovery of PDCD6IP, FASN, XPO1 and ENO1 as new candidate biomarkers for PCa. In the next phase, all proposed candidate biomarkers will be evaluated on patient samples (tissue, serum or urine) to fully elucidate their potential clinical value.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY DATA

Supplementary data for this article are available online at: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0082589

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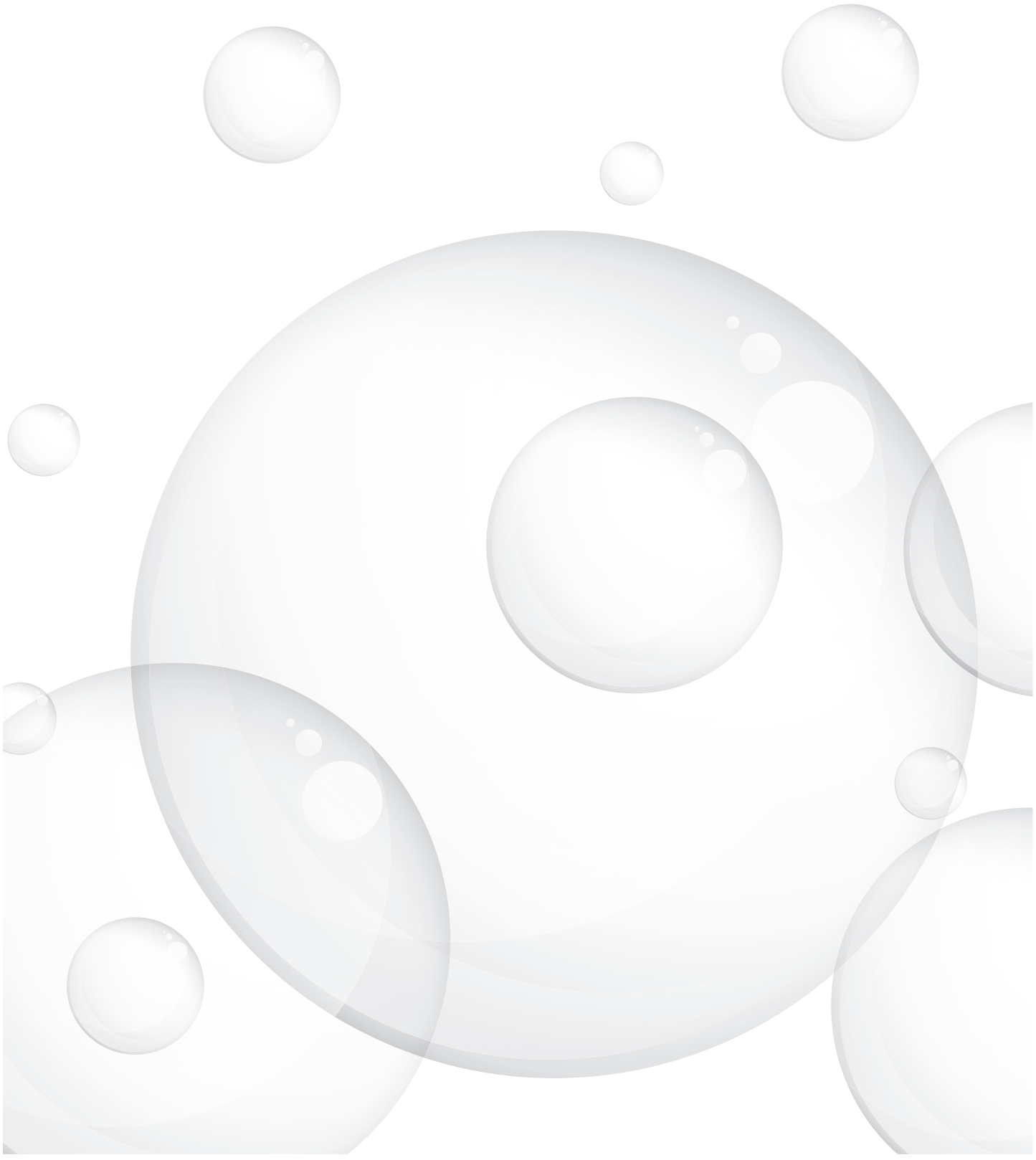

6

Tissue proteomics outlines AGR2 and LOX5 as markers for biochemical recurrence of prostate cancer

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ABSTRACT

Although many patients are cured from prostate cancer (PCa) by surgery only, there are still patients who will experience rising prostate-specific antigen (PSA) levels after surgery, a condition known as biochemical recurrence (BCR). Novel protein prognostic markers in PCa tissue might enable finding better treatment for those patients experiencing BCR with a high chance of metastasis. In this study, we aimed to identify altered proteins in prostate cancer tissue, and to evaluate their potential role as prognostic markers. We used two proteomics strategies to analyse 34 prostate tumors (PCa) and 33 normal adjacent prostate (NAP) tissues. An independent cohort of 481 samples was used to evaluate the expression of three proteins: AGR2, FASN and LOX5 as prognostic markers of the disease. Tissue microarray immunohistochemical staining indicated that a low percentage of positive tumor cells for AGR2 (HR (95% CI) = 0.61 (0.43-0.93), and a low percentage of positive tumor cells for LOX5 expression (HR (95% CI) = 2.53 (1.23-5.22) are predictors of BCR after RP. In contrast, FASN expression had no prognostic value for PCa.

INTRODUCTION

Prostate cancer (PCa) remains to date the most commonly diagnosed cancer in men in the Western world.[1] Although many patients are cured from this disease after radical prostatectomy (RP),[2] one third of patients will show an increment in serum PSA levels -also known as biochemical recurrence (BCR).[3] For those patients, more frequent follow-up and adjuvant therapies are often required to limit progression of the disease,[3,4] There is a high need for robust molecular markers that can distinguish indolent cases of PCa from those that will recur after initial treatment.[3,4]

Small molecules, such as metabolites and lipids, have been associated with the progression of different types of cancer, including PCa.[5] In our previous study, using a LC-MS/MS-based targeted metabolomics method, we found lower concentrations of arachidonic acid (AA) in serum from PCa patients in the most aggressive stage of the disease.[6] In addition, serum levels of hydroxyeicosatetraenoic acid (HETE) metabolites, which are produced by lipoxygenase-type enzymes from AA, were elevated in serum of part of the patients within the same group of advanced PCa.[6] At tissue level, it has been reported that levels of AA in PCa were significantly lower compared to benign prostate tissues.[7] In addition, Yang *et al*. analysed PCa core biopsies and they found that the 15-LOX-2 metabolite 15-HETE, was higher in PCa than in the normal cores.[8] These findings suggest that the AA pathway might play an important role in PCa development and progression. However, analysis of proteins of the AA pathway in PCa, as well as their role in the prognosis of PCa is still unknown. In this study, we used two complementary mass spectrometry approaches to identify differentially expressed proteins in PCa tissue that could be used as prognostic markers for this disease. Protein signatures were validated in an extended cohort using immunohistochemistry on archival PCa tissue and an available tissue microarray. The expression of the selected proteins was evaluated for prediction of biochemical recurrence after radical prostatectomy.

MATERIALS AND METHODS

Clinical Specimens

Discovery set: The protein fractions from tissue RNA isolation of 67 samples (33 NAP tissues and 34 PCa tissues) were analysed (MEC-2004-261); PCa samples were previously published (GSE41408),[9] as well as additional cancerous and control samples, accessible via GEO accession number GSE59745.[10] Clinico-pathologic characteristics of the samples used for proteomics profiling are presented in Supplementary Table 1.

Tissue Microarray (TMA)- Evaluation set: A Tissue Microarray (TMA) was constructed including 481 patients diagnosed with PCa from the European Randomized Study of

Screening for PCa (ERSPC).[11-13] All patients had undergone RP in Erasmus MC between 1987 and 2010, without previous radiation or hormonal therapy. Clinical follow-up was recorded after each control visit at our outpatient clinic, and data were transmitted to a central study database. Post-operative biochemical recurrence (BCR) was defined as an increment of 0.2 ng/mL in serum PSA after two consecutive measurements, with at least three months between measurements. Clinico-pathologic characteristics and follow-up for patients treated by RP are also summarized in Supplementary Table 1.

Sample preparation

Proteomics

The protein interface from tissue RNA isolation with RNA-Bee of 67 PCa tissue samples (33 NAP adjacent tissues and 34 PCa) were kept at -80 °C. For protein digestion, samples were thawed and 50 µL were transferred to a new microcentrifuge tube and precipitated with cold acetone. After spinning down for 10 minutes, the supernatant was removed and the pellet was washed twice with cold acetone. Supernatant was removed and 50 µL 0.1% RapiGest (Waters Corporation, Milford, MA) 50 mM NH_4HCO_3 were added to the protein pellet. The protein pellet was dissolved by external sonication for 5 min at 70% amplitude at a maximum temperature of 25 °C (Digital Sonifier model 450, Branson, Danbury, CT). The proteins were reduced with 10 mM dithiothreitol (DTT) at 60 °C for 30 min. After the mixture was cooled down to room temperature, it was alkylated in the dark with 50 mM iodoacetamide at ambient temperature for 30 min, and digested overnight with 8 µL trypsin 0.1 ug/mL (Promega, Madison, WI). To inactivate trypsin and to degrade the RapiGest, 6 µL of 5% TFA was added and samples were incubated for 30 minutes at 37 °C. Samples were centrifuged at maximum speed for 60 minutes at 4 °C and the supernatant was transferred to a new Eppendorf tube. A fraction of 5 µL was then diluted 40 times and subsequently transferred to LC vials for LC-MS analysis.

Chromatography Separation and Mass Spectrometric Analysis

Samples were measured using a nano-LC system (Ultimate 3000, Thermo Fisher Scientific, Amsterdam, the Netherlands) coupled online to Q Exactive plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Chromatographic and mass spectrometry conditions used are described previously.[14,15] Briefly, 2 µL were injected into the nano-LC after preconcentrating and washing of the sample on a C18 trap column (1 mm×300 µm internal diameter) Thermo Fisher Scientific). Peptides were eluted after loading the sample on to a C18 column (PepMap C18, 75 µm ID × 500 mm, 2 µm particle and 100 Å pore size, Thermo Fisher Scientific) using a linear 90 min gradient (4-25% acetonitrile/H_2O; 0.1% formic acid) at a flow rate of 250 nL/min. The separation of the peptides was monitored by a UV detector (absorption at 214 nm). Data was collected in data-dependent acquisition mode (DDA). Full scan MS spectra (m/z 400-1600) in profile mode were

acquired in the Orbitrap with a resolution of 70,000 after accumulation of an AGC target of 1 x 10^6 using a maximum fill time of 100 ms. The top 12 peptide signals (charge-state 2^+ and higher) were isolated (1.6 Da window) and fragmented by HCD (Higher-energy collision, normalized collision energy 28.0) and measured in the Orbitrap with a AGC target of 50,000, a maximum fill time of 60 ms and a resolution of 17,500. Dynamic exclusion was activated; after the first time a precursor was selected for fragmentation it was excluded for a period of 30 seconds using a relative mass window of 10 ppm. Lock mass correction was activated to improve mass accuracy of the survey scan. Technical replicates of each sample were randomly analysed within the measurement period and no significant changes in the number of identified proteins were observed in time for both the replicates and quality control measurements

Orbitrap-MS/MS Data Processing and Analysis

Label free Quantitation was performed using MaxQuant software (version 1.5.5.1).[16] Data was searched against the UniProt-Swiss-Prot 2014-4 database using the Andromeda[17] search engine incorporated in MaxQuant. Cysteine carbamidomethylation was set as fixed modification and methionine oxidation and N-terminal acetylation were set as variable modifications. Peptide and protein identifications were set at a maximum False Discovery rate of 1%. We used the option "match between runs" option to allow matching identifications across measurements and the minimum number of peptides per proteins required for quantitation was set to 2.

Parallel Reaction monitoring (PRM)

PRM was performed on a nano-LC Fusion Orbitrap system. We used similar settings to the above-mentioned DDA measurements on the nano-LC, with the difference of an elution gradient of 60 min. A targeted MS/MS method was developed for 37 peptides, as presented in Table 2. A quadrupole isolation window of 1 m/z units, an AGC target of 2e5 ions, a maximum fill time of 50 ms and an orbitrap resolving power of 240,000 at 200 m/z were used. A fixed HCD normalized collision energy for all peptides of 27 was used, retention times were determined for all peptides, using multiple injections of a tissue sample in which a signal for all 27 peptides was present. Based on the determined retention times a scheduled method was established using a 3 minutes retention time window for each peptide.

Immunohistochemistry

Tissue slides (5 µm) were mounted on aminoacetyl-silane coated glass slides (Statfrost, Berlin, Germany), deparaffinised in xylene and dehydrated in ethanol. Endogenous peroxidase was blocked by 1% hydrogen peroxide in methanol for 20 min. Samples were pretreated by microwave (700 W) in TRIS-EDTA pH 9.0 or in citrate buffer pH 6.0 for 15

min. The slides were incubated overnight at 4 °C with the following primary antibodies targeting anterior gradient protein 2 (AGR2; 1:100; HPA007912, Sigma); fatty acid synthase (FASN; 1:400; ab22759, Abcam, Cambridge, MA, USA); arachidonate 15-lipoxygenase type B (LX15B 1:2000, ab23691, Abcam, Cambridge, MA, USA), and arachidonate 5-lipoxygenase (LOX5; 1:200 ab169755, Abcam, Cambridge, MA, USA).[13] Chromogenic visualization was performed with the EnVision DAKO Kit (Dako, Glostrup, Denmark). After counterstaining with haematoxylin, slides were thoroughly washed, dehydrated, cleared in xylene and mounted in malinol (Chroma-Geselschaft, Körgen, Germany).

In the tissue microarray, immunohistochemical staining for AGR2, 5-LOX and FASN was visually examined as described previously.[18] Staining intensity was scored as negative (0; no staining), weak (1+; only visible at high magnification), moderate (2+; visible at low magnification), and strong (3+; striking at low magnification). If there was heterogeneous expression, the strongest intensity was used for further analyses. For AGR2, the percentage of positive tumor cells was counted and used for further analyses. For optimization and validation of all immunohistochemical procedures we used appropriate internal and external controls, and omitted first antibodies to exclude non-specific binding.[19]

Statistics

Protein annotation and statistical testing for differences (two-sided Student's T-test, permutation-based FDR 0.05) in the proteomics shotgun experiments was performed in Perseus.[20] Protein normalised intensities were log2 transformed before testing. The PRM data were analysed using Skyline version 3.5.0.9320 MacCoss Lab Software, Seattle, WA; fragment ions for each targeted mass were extracted and peak areas were integrated. Data matrix from PRM experiment was processed in GraphPad Prism 5 for Windows and R version 3.2.3. One-way Analysis of Variance (ANOVA) allowing multiple comparisons was used to estimate differences among Gleason score groups.

Associations between clinico-pathologic parameters and protein expression in TMA experiments were performed by student t-test or chi-squared test. Survival curves were calculated according Kaplan-Meier (KM), and to detect significant survival differences the Log-Rank test was used. Univariate and multivariate Cox regression were used to determine predictive properties of AGR2, LOX5 and FASN for BCR. A two-sided p-value of ≤ 0.05 was considered significant. Statistical analysis for the TMA expression was performed in SPSS version 22.

RESULTS

Proteomics

In this study we aimed to find protein signatures of PCa with potential applicability towards prognosis of the disease. To identify differentially expressed proteins in PCa, we used shotgun proteomics using the protein fraction from RNA-bee isolation of 34 PCa and 33 NAP tissues (Figure 1). Using label free quantification (LFQ), a total of 2865 proteins were identified, and 798 proteins were statistically significant (FDR<0.01). Figure 2a illustrates the LFQ mean ratio between PCa and NAP, also indicating that an elevated number of proteins were up-regulated in PCa. The list of identified proteins as well as the differentially expressed proteins in PCa is presented in Supp. Table 2. Two proteins, anterior gradient 2, AGR2, and fatty acid synthase, FASN, were highly up-regulated in PCa and their normalised abundances showed also a trend when compared with the Gleason score, as shown in Figure 2b-c. These two proteins were considered for further analysis.

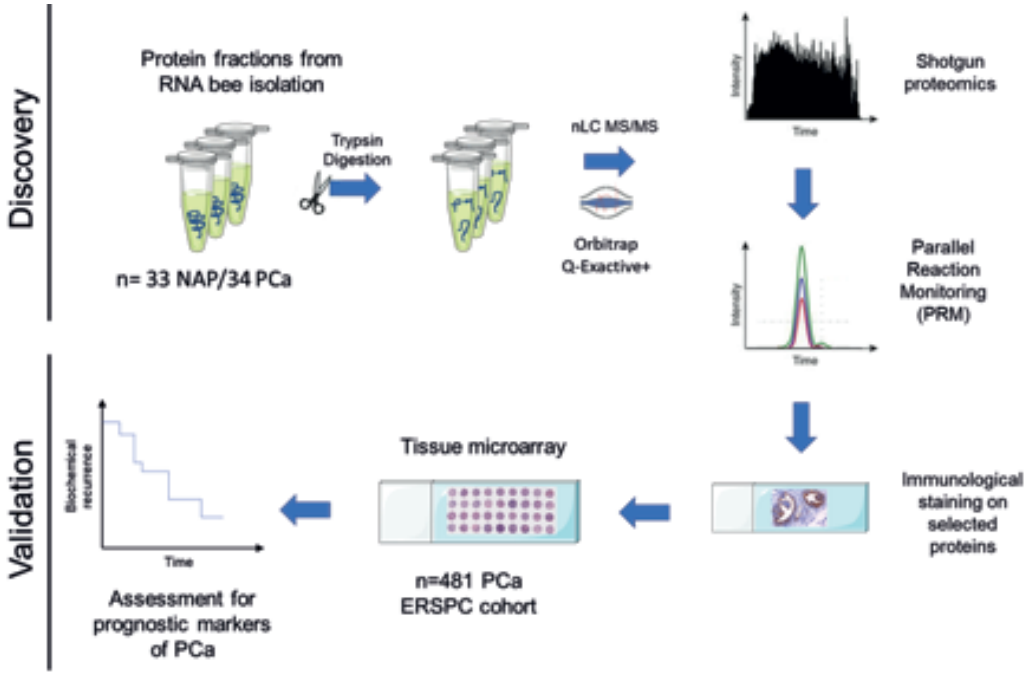

Figure 1. Schematic representation of the identification of markers associated to biochemical recurrence of PCa. Protein fractions from RNA isolation of PCa tissue were digested and analysed by nano-LC-MS/MS using both shotgun and PRM modes. Four selected proteins were validated by immunohistochemistry and three proteins: AGR2, FASN and LOX5, were analysed using TMA staining. Kaplan-Meier were constructed to evaluate the prognosis of the markers.

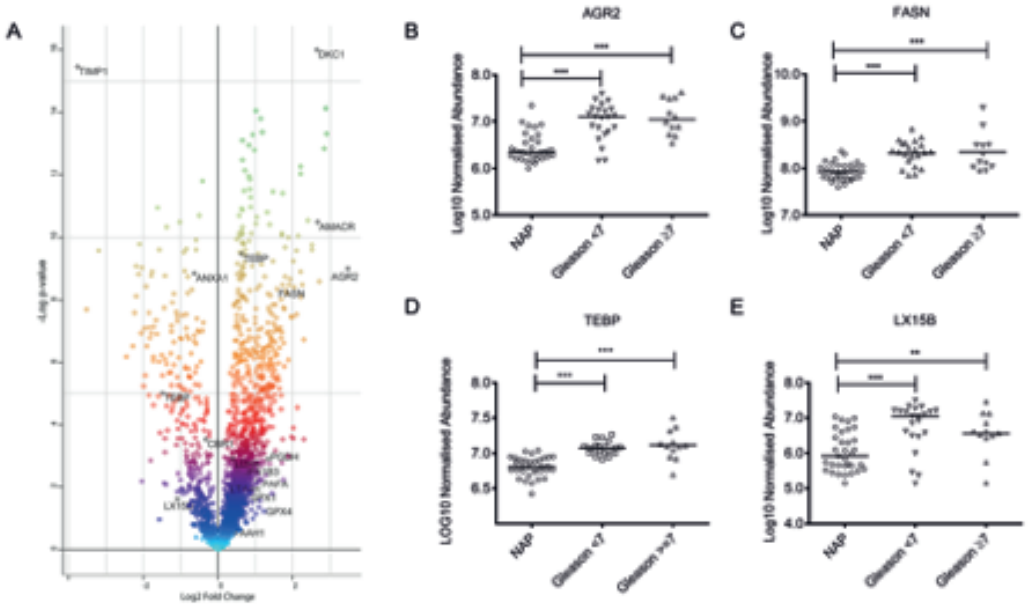

Figure 2. A Volcano plot illustrating the proteins identified in the shotgun proteomics approach. Non-differentially abundant proteins are colored in blue whereas statistically significant proteins are colored from red up to green (lowest p-value) Proteins belonging to the AA pathway are labelled in black letters. B-E Boxplots of normalized abundances at different Gleason scores for proteins AGR2, FASN, TEBP and LX15B. **B-E** Boxplots of normalized abundances at different Gleason scores for proteins AGR2, FASN, TEBP and LX15B.

To analyse whether proteins in the Arachidonic acid (AA) pathway could be used as prognostic markers for PCa, we manually searched the list of differentially expressed proteins and compared this list with the list of 79 proteins in the AA pathway as described by Sabidó *et al.* (Supp Table 3).[21] Interestingly, we identified fifteen proteins of the AA pathway in the list of de-regulated proteins, and particularly, prostaglandin E synthase 3. TEBP, showed a degree of correlation with the Gleason score, as is shown in Figure 2D.

We previously reported high levels of HETE metabolites in serum from PCa patients. In our shotgun proteomics dataset, we only identified one lipoxygenase-type enzyme deregulated in PCa tissue: LX15B, but its expression is higher in NAP and the statistical test is significant ($p<0.05$), as shown in Figure 2E. The deregulated proteins in PCa which are part of the AA pathway are presented in Figure 2A.

Gleason score, pT Stage and ERG oncogene status have been associated with poor prognosis of PCa. To evaluate whether proteins identified in PCa tissue could be associated with these clinical parameters, we performed statistically tests on patients with different Gleason scores (GS 6 vs. GS 7), different pT stages (pT2, pT3, pT3) and whether ERG is activated or not (ERG oncogene-positive vs. ERG oncogene-negative). We did not find statistically significant proteins when comparing Gleason score or pT stage, but we

found that 15 proteins were statistically significant when comparing ERG activation. Interestingly, three of these proteins belonging to the AA pathway and were up-regulated (FDR<0.01): phospholipase A2, PA2GA, arachidonate 15-lipoxygenase B, LX15B, and prostaglandin reductase 1, PTGR1. The list of differentially expressed proteins when ERG is activated is presented in Supp. Table 4. These results indicate that the AA pathway might play a role in ERG activation.

Table 1. A. Clinico-pathologic correlations in the PCa-TMA and AGR2 (percentage of positive tumor cells). Positive =100% positive cells, Negative = less than 100% positive tumor cells.

	Negative	**Positive**	**Total**	***p-value***
PSA at diagnosis				
≤10 ng/ml	88 (21.8%)	264 (65.3%)	352 (87.1%)	0.141
>10 ng/ml	18 (4.4%)	34 (8.5%)	52 (12.9%)	
Total	106 (26.2%)	298 (73.8%)	404	
Gleason score				
<7	44 (10.9%)	170 (42.0%)	214 (52.9%)	0.017
7	51 (12.6%)	112 (27.6%)	163 (40.2%)	
>7	11 (2.7%)	17 (4.2%)	28 (6.9%)	
Total	106 (26.2%)	299 (73.8%)	405	
pT-stage				
pT2	68 (16.8%)	212 (52.3%)	280 (69.1%)	0.121
pT3/4	38 (9.4%)	87 (21.4%)	125 (30.9%)	
Total	106 (26.2%)	299 (73.8%)	405	

B. Intensity of positive tumor cells, Negative = weak or no staining, Positive = strong staining intensity.

	Negative	Positive	Total	*p-value*
PSA at diagnosis				
≤10 ng/ml	9 (2.2%)	343 (84.9%)	352 (87.1%)	0.203
>10 ng/ml	3 (0.8%)	49 (12.1%)	52 (12.9%)	
Total	12 (3.0%)	392 (97.0%)	404	
Gleason score				
<7	2 (0.5%)	212 (52.3%)	214 (52.8%)	0.032
7	8 (2.0%)	155 (38.3%)	163 (40.3%)	
>7	2 (0.5%)	26 (6.4%)	28 (6.9%)	
Total	12 (3.0%)	393 (97.0%)	405	
pT-stage				
pT2	9 (2.3%)	271 (66.9%)	280 (69.2%)	0.465
pT3/4	3 (0.7%)	122 (30.1%)	125 (30.8%)	
Total	12 (3.0%)	393 (97.0%)	405	

Table 2. Predictive value of protein marker expression for biochemical recurrence (BCR) after radical prostatectomy (RP).

	Univariate Analysis			Multivariate Analysis		
	HR	**(95% CI)**	**P-Value**	**HR**	**(95% CI)**	**p-value**
Age	1.06	(1.01-1.10)	0.02	0.97	(0.91-1.04l)	0.46
PSA concentration	3.38	(2.22-5.16)	<0.01	1.38	(0.68-2.82)	0.38
Gleason Score	2.66	(2.00-3.54)	<0.01	2.39	(1.47-3.85)	<0.01
pT-Stage	1.74	(1.47-2.05)	<0.01	1.30	(0.99-1.72)	0.06
Surgical Margins	3.09	(2.12-4.50)	<0.01	1.70	(0.90-3.17)	0.10
AGR2 Percentage of positive tumor cells	0.61	(0.43-0.93)	0.02	1.10	(0.60-2.01)	0.77
LOX5 Percentage of positive tumor cells	2.53	(1.23-5.22)	0.01	2.30	(1.08-4.98)	0.03
FASN intensity	0.84	(0.47-1.47)	0.55	-	-	-

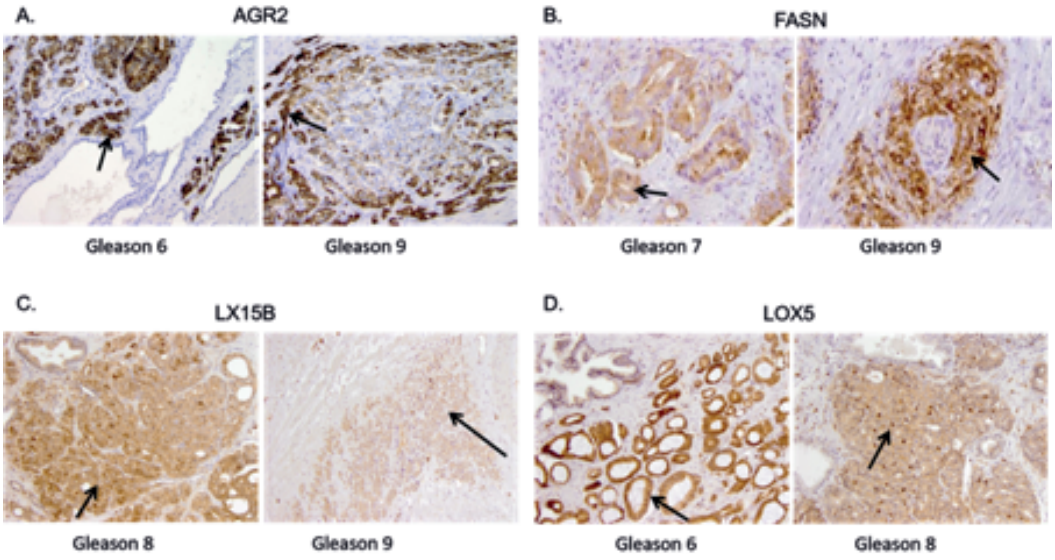

Figure 3. Immunohistochemical staining in PCa tissue for A. Anterior Gradient 2 (AGR2) in Gleason score 6 and Gleason score 9, B. Fatty Acid Synthase (FASN) in Gleason 7 and Gleason 9, C. 15-lipoxygenase-2 (15LXB) in Gleason score 8 and Gleason 9 and D: 5-lipoxygenase (LOX5) Gleason 6 and Gleason 8. Arrows indicate stating in positive cells.

To validate the results obtained in the shotgun proteomics experiment, we performed parallel reaction monitoring measurements (PRM) on the same samples described above, and we included other proteins not identified by shotgun approach in order to evaluate their potential as prognostic markers for PCa. We selected these proteins (Table 2) because they are involved in the metabolism of small molecules and fatty acids (polyamines, eicosanoids and phospholipids), as well as in the so-called Warburg effect, which also involves metabolic enzymes of metabolites present in the TCA cycle.[22,23] In total we analysed 18 proteins and each protein was quantified using two peptides.

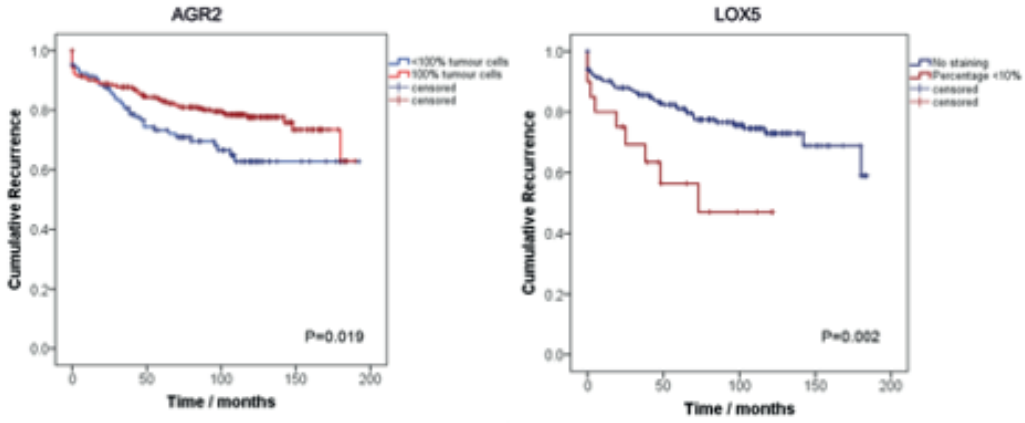

Figure 4. Kaplan-Meier curves assessing the probability of PCa biochemical recurrence after radical prostatectomy by A: AGR2 and B: LOX5. Blue lines represent: AGR2: Percentage of tumor cells <100% B: LOX5: No staining. Red lines represent: AGR2: 100% of positive tumor cells, LOX5: <10% staining of positive tumor cells.

We included two peptides for PARK7, a protein recently described for normalisation of proteomics experiments,[24] and as expected, peak areas between samples were not statistically significant ($p>0.05$) different.

AMACR, a known marker for PCa, and one of the proteins with the highest fold change and statistically significant different in the shotgun experiments, was included as positive control for the PRM measurements. ANOVA comparison between NAP and PCa at different Gleason Scores indicated a high up-regulation in PCa ($p<0000.1$) for AMACR, confirming the validity of our PRM set up. We could also confirm the shotgun results of AGR2 and FASN proteins using PRM, in both cases the peak area and the Gleason score correlated well. A summary of the statistical calculations performed for the 18 proteins analysed by PRM is presented in Supplementary Table 5.

To validate the importance of the AA pathway in PCa and its possible application in prognosis, we analysed seven AA pathway proteins by PRM. We included LOX5 as it is involved in the production of eicosanoid-like compounds, and it was not identified by shotgun. Statistical analysis presented in Supp. Table 5 indicated that LOX5 and TEBP are highly up-regulated in PCa tissue and they are also correlate with the Gleason score. On the other hand, LX15B, HYES, PGH1 were down-regulated (ANOVA, $p<0.001$) and LKHA4 and FAAH were not different when compared with the Gleason score.

To identify whether selected proteins involved in metabolic reprogramming (Warburg effect) are de-regulated in PCa, we included transitions for another seven proteins in the PRM setup. Interestingly, the ANOVA test indicated that ACSL3, GLSK, LDHA, LDHB and ANM1 proteins are statistically significant ($p<0.001$). Two proteins involved in polyamine metabolism, SMS and SRM, were not different between NAP and PCa, even though this pathway is known to be heavily deregulated in PCa.[22]

Immunohistochemistry

Based on the results of the mass spectrometry experiments we selected four proteins for immunohistochemical (IHC) validation: AGR2, FASN, LOX5 and LX15B. These proteins were selected based on their correlation with the Gleason score and the availability of antibodies. In addition, these antibodies ought to work reliably using formalin-fixed paraffin-embedded (FFPE) tissue. IHC staining of the selected proteins on PCa FFPE tissue sections is shown in Figure 3.

Figure 3. Immunohistochemical staining in PCa tissue for A. Anterior Gradient 2 (AGR2) in Gleason score 6 and Gleason score 9, B. Fatty Acid Synthase (FASN) in Gleason 7 and Gleason 9, C. 15-lipoxygenase-2 (15LXB) in Gleason score 8 and Gleason 9 and D: 5-lipoxygenase (LOX5) Gleason 6 and Gleason 8. Arrows indicate stating in positive cells.

AGR2 showed heterogeneous expression in normal luminal epithelium and PCa. AGR2 staining was strikingly positive in cancer and negative in normal (Figure 3A). Expression of cytoplasmic FASN was negative to weak and rarely moderate in normal prostate luminal epithelium. Expression in PCa was stronger (1+/2+) than in adjacent normal tissue (0/1+) with locally strong expression (3+) in Gleason grade 7 and 9 areas (Figure 3B).

Expression of LX15B was generally moderate to strong (2+/3+) and occurred in both cytoplasm and nucleus of both benign luminal cells and PCa. Normal basal epithelium and atrophic prostate epithelium generally showed lower expression (0/1+). Stromal expression was negative (0) to weak (1+) (Figure 3C).

LOX5 staining was found to be predominantly expressed in the nuclei and cytoplasm of benign basal epithelial cells and atrophic luminal epithelial cells (1+ to 3+). Normal luminal epithelial cells were generally negative (0) or weakly positive (1+). PCa showed enhanced expressions as compared to benign luminal cells varying from weak to strong, but no clear association with the Gleason score was observed (Figure 3D).

Tissue Microarray

To determine whether expression of AGR2, FASN, and LOX5 might correlate with clinical parameters we analysed these proteins in 481 samples from RP patients. Cytoplasmic expression of AGR2 occurred in 84% (404/481) of the patients, with 52% of cores showing strong intensity (3+). 74% (299/404) of the cores exhibited staining in 100% of tumor cells. Strong FASN staining occurred in 86% (399/461) of patients. We did not find any expression of LOX5 in 54% of the cores, and both the cytoplasmic and the nuclear intensities were weak (1+) in most cases. The percentage of positive tumor cells stained for LOX5 was lower than 10% in the 224 positive cores. An association between the Gleason score (GS) and the percentage of positive tumor cells and intensity of AGR2 was found ($p=0.017$, and $p=0.032$, respectively, as described in Table 1A-B). AGR2 expression occurred more often in patients with lower GS (42% in patients with GS <7 when analysing percentage of tumor cells, and 52.3% when cytoplasm was analysed). FASN expression

was higher in GS <7 and GS=7 (49.0% and 34.6% respectively) than in GS>7 (5.4%), but no correlation existed between FASN and PSA, GS or pT stage (Supplementary Table 6). A correlation between pT stage and cytoplasm intensity of LOX5 was found (*p= 0.044,* in Supplementary Table 7A). No other correlation was found when analysing cytoplasmic intensity, nuclear intensity, or percentage of positive tumor cells for LOX5 (Supplementary Table 7 B-C).

We constructed Kaplan Meier (KM) curves to identify the role of AGR and LOX5 in predicting BCR after surgery. We analysed if the percentage of positive cells of AGR2 100% (positive) or lower than 100% (any negative) was predictive for BCR. We found that a percentage lower than 100% of positive tumor cells (<100%) in AGR2 was predictive for BCR (HR (95% CI) = 0.61 (0.43-0.93); *p=0.02*), as described in Table 2 and Figure 4.

For LOX5, we analysed whether the percentage of positive cells was negative (LOX5 0%), or it had any expression (LOX5 > 0%). Expression for LOX5 was characterised by only a small percentage of positive tumor cells, being at maximum 10%. KM curves indicate that low percentage of LOX5 positive tumor cells is a predictor of BCR in comparison with negative staining (0%), in a univariate analysis (HR (95% CI) = 2.53 (1.23-5.22); *p*=0.002), as presented in Table 2 and Figure 4.

DISCUSSION

The Gleason score is an effective indicator of aggressiveness of PCa and therefore an important parameter to determine prognosis. However, more knowledge of which patients will relapse after radical prostatectomy and/or which patients will respond better to a specific treatment is still needed. AGR2 is a predictor of biochemical recurrence after performing TMA immunostaining in the Evaluation set (Figure 4), confirming previous reports for this protein as biomarker for PCa.[25-27] Bu *et al*., demonstrated that AGR2 is overexpressed in PCa, particularly in low-grade tumors and also in tumor precursor lesions PIN. In addition, high levels of AGR2 transcript were found in urine sediments from PCa patients.[28] Two distinct splice variants of AGR2 in urine exosomes have been identified as effective markers distinguishing NAP and PCa.[29] AGR2 has been reported to be induced by androgens in PCa,[30] and its tumorigenic function is associated with cell growth, survival and metastasis, as recently reviewed.[31]

Fatty acid synthase (FASN) is known to be a key enzyme in the production of long chain fatty acids from Acetyl-CoA and Malonyl-CoA.[32] Overexpression of this protein in PCa tissue has been reported in cell lines[33], tissue microarrays[34], tissue biopsy cores[35] and exosomes.[36] FASN-normalised intensity was high in PCa in our proteomics dataset and its expression was independently evaluated by immunohistochemistry and a TMA. Although its expression does not predict biochemical recurrence, inhibition of FASN

has been proposed as a therapeutic target because of its increased expression and its relation to both cell cycle arrest and apoptosis.[37] Our results reinforce the theory that FASN could be an important target to manipulate the fatty acid and lipid metabolism in cancer and therefore control cancer cell behaviour.[38-40]

The AA pathway is a key inflammatory pathway involved in cellular signalling as well as prostate carcinogenesis.[41] Arachidonic acid is stored in cell membranes as a phospholipid, it is released by the action of phospholipase A2-type enzymes, and then metabolised by the action of cyclooxygenases (COX), lipoxygenases (LOX) and P450 cytochromes to produce biologically active eicosanoids.[8]

We found that the abundance of lipoxygenase 15 type 2 (LX15B), an enzyme encoded by the gene *ALOX15B*, was lower in PCa than in NAP. Evaluation by immunohistochemistry showed a moderate increased abundance in both cytoplasm and nucleus of normal luminal cells and PCa when compared to normal basal epithelium. These results do not support our hypothesis that the previously reported high serum concentration of HETE metabolites could be explained by an up-regulation of this lipoxygenase-type enzyme.[6] Interestingly, low expression of LOX5 in PCa tissue is slightly higher in PCa compared to NAP, and this expression can be used to evaluate BCR after surgery (Figure 4). In addition, we noticed that expression of upstream enzymes, such as the phospholipases (PA2PA), is higher in PCa when there is an activation of ERG-oncogene (Supp. Table 4). These results highlight the importance of the AA pathway in PCa, and, particularly, when ERG is activated. However, functional studies need to be performed in order to analyse the link between ERG activation and the de-regulation of enzymes in the AA pathway, as well as the HETE metabolites or other eicosanoid-type fatty acids in the development of PCa.

Association of different enzymes of this family with PCa has recently been described in literature . Patel *et al,*[42] studied the expression of cytosolic phospholipase A_2 in PCa cells and they reported that increased levels of this enzyme were observed in androgen-insensitive PCa cell lines and they suggested that this enzyme plays a role in cancer cell proliferation and apoptosis. PAFAH (*PLA2G7*) enzyme was identified by Vainio *et al*. in a set of 9783 human tissue samples and it was proposed as a potential drug target specially in ERG positive PCa.[43] Validation studies performed by the same group indicated a correlation between staining intensity for PAFAH and Gleason Score in 50 % of the cases, thus suggesting that both enzymes can be seen as biomarkers for PCa, and the PAFAH inhibition by statins as a therapeutic tool for managing the disease.[41]

We found that the protein TEBP (*PTGES3*), was up-regulated in PCa tissue, TEBP protein is involved in eicosanoid signalling as it produces the Prostaglandin E2, involved in inflammation processes. In addition, it is reported to be an enhancer of androgen receptor activity. It is involved in AR binding to chromatin, which is a critical step in AR signalling and PCa development.[44,45] Further validation is still required, using both quantitative

mass spectrometry and immunohistochemistry, to confirm a potential role of this protein in PCa diagnosis and prognosis. In addition, further analysis *in-vitro*, could address the role of the metabolite prostaglandin E, produced by TEBP along the AA pathway in PCa development and progression.

Cancer cells demand energy for proliferation and therefore there might be a metabolic reprograming in the cancer progression process (Warburg effect). By using both shotgun and PRM experiments, we noticed that some metabolic enzymes are de-regulated in PCa (Supp. Table 5). It is of interest that the expression of lactate dehydrogenase-A protein (LDHA) was significantly ($p<0.0001$) lower in PCa than in NAP, and it is also de-regulated when ERG is activated (Supp. Table 4). This result might be associated to previous reports for this protein indicating a key role in PCa oncogenesis.[46] LDHA executes the final step of aerobic glycolysis and has been reported to be involved in tumor progression.[47] It was recently demonstrated that LDHA overexpression is highly linked to local relapse of PCa.[48]

In conclusion, the experiments in this study allowed the identification of proteins and pathways associated to PCa. We identified a relationship between proteins in the AA pathway and PCa, and we have shown that expression of LOX5 and AGR2 in tissue predict biochemical recurrence after radical prostatectomy. Further validation studies on independent cohorts using different antibodies are needed to analyse the role of TEBP in PCa progression, as well as their clinical applicability. In addition, functional analyses are still required to fully understand their role in cancer cell proliferation, apoptosis and senescence.

ACKNOWLEDGMENTS

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SUPPLEMENTARY DATA

Supplementary data for this article are available online at: https://doi.org/10.18632/oncotarget.26342

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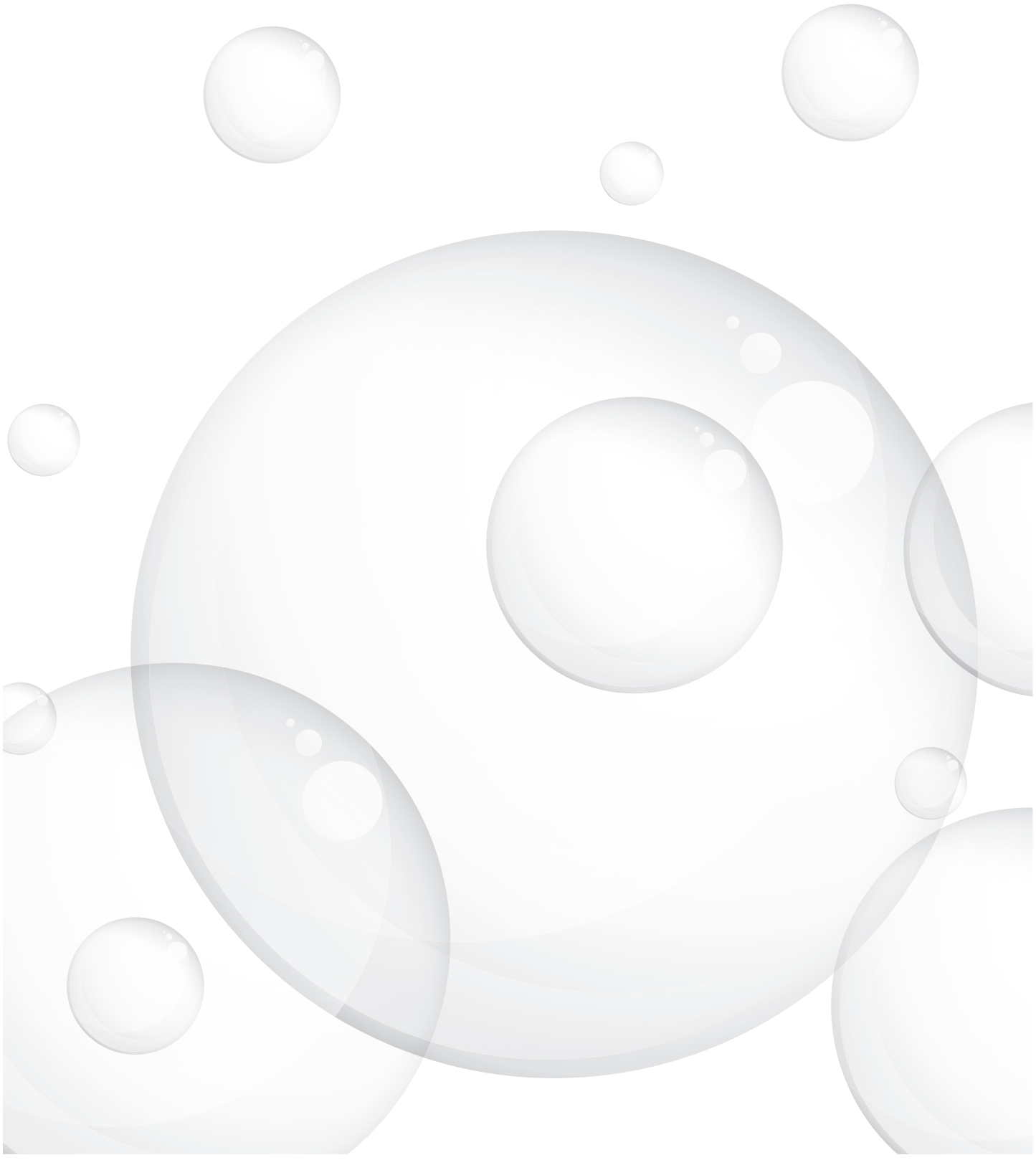
PART 2

Development of an extracellular vesicle-based assay



7

Immuno-based detection of extracellular vesicles in urine as diagnostic marker for prostate cancer

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ABSTRACT

Introduction

Extracellular vesicles (including the subclass exosomes) secreted by cells contain specific proteins and RNA that could be of interest in determining new markers. Isolation/characterization of PCa-derived exosomes from bodily fluids enables us to discover new markers for this disease. Unfortunately, isolation with current techniques (ultracentrifugation) is labor intensive and other techniques are still under development. The goal of this study was to develop a highly sensitive time resolved-fluorescence immunoassay (TR-FIA) for capture/detection of PCa-derived exosomes.

Material and methods

In our assay, biotinylated capture antibodies against human CD9 or CD63 were incubated on Streptavidin-coated wells. After application of exosomes, Europium-labeled detection antibodies (CD9 or CD63), were added. Cell medium from 37 cell lines was taken to validate this TR-FIA. Urine was collected (after digital rectal exam) from patients with PCa (n=67), men without PCa (n=76). As a control, urine was collected from men after radical prostatectomy (n=13), women (n=16) and patients with prostate cancer without digital rectal exam (n=16). Signal intensities were corrected for urinary PSA and creatinine.

Results

This TR-FIA can measure purified exosomes with high sensitivity and minimal background signals. Exosomes can be measured in medium from 37 cell lines and in urine. DRE resulted in a pronounced increase in CD63-signals. After DRE and correction for urinary PSA, CD9 and CD63 were significantly higher in men with PCa.

Conclusions

This TR-FIA enabled us to measure exosomes with high sensitivity directly from urine and cell medium. This TR-FIA forms the basis for testing different antibodies directed against exosome membrane markers to generate disease-specific detection assays.

INTRODUCTION:

Prostate specific antigen (PSA, KLK3) is a protein that is commonly used in daily practice to aid urologists in diagnosing prostate cancer (PCa). Although PSA has a high sensitivity, it lacks specificity and therefore causes unnecessary biopsies. Furthermore, PSA is a poor prognostic marker.[1] In order to increase specificity and distinguish between the clinically insignificant cancers and the ones that are clinically relevant, novel markers have to be identified. Recent studies have shown that extracellular vesicles and particularly the vesicles from endosomal origin, referred to as exosomes, could help us in identifying novel tissue-specific markers. Moreover, also their presence and number might be indicative of disease.[2-4]

Quantifying the number of exosomes and characterizing them on single particle level remains challenging. To determine the number of exosomes in body fluids or measure an exosomal marker of interest, purification and concentration steps are often needed. Isolation of exosomes is most commonly performed by ultracentrifugation, filtration, precipitation or antibody-based capture technologies. Most of these protocols are still under development, labor intensive and limited with respect to efficient isolation or purity of the final exosomal preparation.[5] Measuring the number and size distribution of the exosomes is a next challenge and technologies such as nanoparticle tracking analysis, tunable resistive pulse sensing and flow cytometry (FACS) are utilized, although again, each with their own set of limitations.[6]

In order to count exosomes or measure an exosomal protein marker in bodily fluids in a clinical setting, novel or optimized assays have to be developed. One technique that is of special interest is an immunoaffinity assay (e.g. ELISA or TR-FIA) directed against exosomal transmembrane proteins. With a sandwich-structure, exosomes can be captured without cumbersome preprocessing from body fluids (e.g. plasma, serum or urine) and characterized with antibodies directed against disease-specific markers. Although the quantity of the protein of interest is reliably measured, the immunoassays in this format do not provide an exact number for the concentration of exosomes.

Current efforts to establish such an immunoassay have room for improvement.[7-9] Published reports have shown a sandwich ELISA (CD9-based) with which it is possible to isolate and detect exosomes from plasma.[7] Exosomes had to be purified first and the minimum amount of exosomes that could be detected was 3-10 µg exosomal protein. We developed a TR-FIA (time resolved-fluorescence immunoassay) against the transmembrane proteins CD9 and CD63. Both proteins are known to be commonly expressed on the membrane of exosomes derived from different cell types. Antibodies against these proteins were biotinylated for optimal capture and another batch labeled with a Europium-chelate for time resolved fluorescence detection. As compared to ELISA, the TR-FIA typically has a higher sensitivity and dynamic range and does not require an en-

zymatic reaction.[10] With this TR-FIA we showed that extracellular vesicles can directly be measured in cell medium and urine and can be used as a marker for the presence of PCa.

MATERIAL AND METHODS

Cell Culture

Prostate cancer cell lines (DU145, VCaP, PC3 and LNCaP), two immortalized prostate epithelial cell lines (PNT2C2 and BPH-1) and the hepatocellular carcinoma cell line (Hep3B) were used for exosome isolation. All cell lines were cultured in ten T175 (175 cm2) culture flasks (Greiner Bio-One, Frickenhause, Germany) up to 80-100% confluency. DU145 (androgen independent)[11], LNCaP (androgen dependent)[12], PC3 (androgen independent)[13] and PNT2C2[14] were cultured in RPMI 1640 (Lonza, Verviers, Belgium) and supplemented with 5% fetal calf serum (FCS) and 500 U penicillin/ 500 U streptomycin (P/S). BPH-1[15] and VCaP[16] (androgen dependent) were cultured in the same medium and supplements, only with 10% FCS. Hep3B[17] was cultured in Alpha MEM (Lonza) supplemented with 5% FCS and P/S. When 80-100% confluency was reached, cells were 48 h incubated with FCS-free medium. Cell medium was collected for exosomes isolation. All other cell lines tested were cultured under their optimal conditions in regular medium, containing various concentrations of serum. When 80-100% confluency was reached, cell medium was centrifuged 3000 x g and the supernatant stored for short term at 4˚C or long term at -80˚C.

Urine collection

Whole urine from men with PCa (n=67) and without PCa (n=76) was collected at the out patients clinic from the Erasmus Medical Center Rotterdam after written consent (Medical Ethics Approval number 2005-077 and 2010-176). Urine samples from men were collected after digital rectal exam (DRE) at the time of day most convenient to the person. DRE was performed to increase prostate fluid secretion and therefore the number of prostate-derived vesicles in the urine. From another 16 men with prostate cancer, urine was collected without DRE. From each prostate cancer patient, PSA-levels and prostate biopsy results (Gleason score) are known. Gleason scores varied from 6 (3+3) up to 9 (5+4). Furthermore, whole urine from women (n=16) and men after radical prostatectomy (n=12) was collected at the person's convenience and were used as controls. Patient characteristics are shown in Table 1. All urine samples were centrifuged at 3,000 x g (20 min) in order to remove cellular debris. Subsequently, urine samples were stored in 1.5 mL aliquots at -80 ^{0}C.

Isolation of exosomes

Exosomes were isolated according to a protocol that was previously described.[4,18] Briefly, cell culture medium was subjected to consecutive centrifugation steps (3,000 x g and 10,000 x g) to remove cellular debris and large vesicles. Exosomes were then pelleted with ultra-centrifugation at 64,000 x g (2 h) and at 100,000 x g (1 h, in a 0.32 M sucrose solution). Sucrose supernatant was removed and pellets resuspended in 100 µL PBS and stored at -80 ^{0}C. Total amount and concentration of exosomal proteins was measured with a BCA-assay (Pierce, Rockford, IL, USA).

Time resolved fluorescence immunoassay (TR-FIA)

Biotinylation of capture antibodies

A streptavidin-coated 96-well plate (KaiSA96, Kaivogen, Turku, Finland) was used to bind biotinylated capture antibodies. CD9 (mouse monoclonal against human CD9, clone MAB1880, R&D systems, Abingdon, UK) and CD63 (mouse monoclonal against human CD63, clone 556019, BD Bioscience, Breda, Netherlands) antibodies were biotinylated.[19] Biotin isothiocyanate (BITC) was dissolved in ethanol to a final concentration of 10 mM. Before adding biotin, the antibody solution had to be adjusted with 0.5 M carbonate buffer to a pH of 9.8. For the most optimal final concentration of 2 mg/ml biotinylated antibodies, a 40-fold excess of biotin was used. Antibodies and BITC were incubated for 4 hours at room temperature. Unreacted BITC was removed by gel filtration with a NAP-5 column (GE-Illustra, Diegem, Belgium).

MaxiSorp plates (Thermo Scientific, Amsterdam, Netherlands) were used to bind exosomes directly to the plate (without capture antibodies) or to capture exosomes with unbiotinylated antibodies (CD9/CD63).

Labeling detection antibodies with Europium (Eu)

Antibodies used for Eu-labeling were CD9 (same as above), CD63 (same as above), and Human Kallikrein 2 (KLK2). 2 mg Eu-chelate (Perkin-Elmer, Turku, Finland) was dissolved in 200 µL sterile water and filtered through a 0.22 µm filter. From this, 100 nM Eu-chelate was dissolved in Enhancement solution (Product number 1244-105, Perkin-Elmer) to reach 1 nM. For optimal results the final concentration of labeled antibodies should be 2 mg/ml. A 100-fold excess of Eu-chelates was added to the antibody. pH was adjusted to 9.8 with 0.5 M carbonate buffer. The antibodies and Eu-chelates were incubated overnight at 4 ^{0}C. Purification of the labeled antibodies was performed by gel filtration (FPLC), using a Superdex 200 column (GE Healthcare Europe, Helsinki, Finland) with a flow rate of 30 ml/h. Fractions containing the protein were pooled. BSA was added to a final concentration of 0.1% and filtered through a 0.22 um filter and stored at 4 ^{0}C.

TR-FIA protocol

The streptavidin-coated plates were incubated with 200 ng biotinylated CD9 or CD63 in 100 µL per well for 1 h with shaking (750 rpm) at room temperature. Supernatant was removed and the plates were washed with Wash buffer (Product nr 42-02, Kaivogen, Turku, Finland) three times with an automatic plate washer (TECAN Columbus). Subsequently, samples (in triplicate) were diluted in the sample buffer and added to the wells and incubated for 1 h with shaking at room temperature. The plates were washed again three times. 25 ng of Eu-labeled antibodies was added per well (suspended in 100 µL sample buffer) and incubated for 1 hour at room temperature. Excess antibody was removed and the plates washed again three times. 100 µL Enhancement solution (Perkin-Elmer) was added and incubated for 15 min at room temperature with slow shaking. Europium (time resolved fluorescence) was measured by the Wallac Victor 2, 1420 multilabel counter (Perkin-Elmer) at a wavelength of 615 nm. For analysis of the patient samples the assay was constructed with either biotinylated CD9 with Europium-labeled CD9 (CD9-assay) or the combination of biotinylated CD63 with Europium-labeled CD63 (CD63-assay).

LNCaP cell culture medium was collected in a large batch, centrifuged at 3000 x g, aliquoted and stored at -80˚C for the 0, 25, 50 and 100 µL control concentration series for each 96-well plate we performed. These concentration series were used to correct signal level variability between plates.

Urinary PSA and creatinine

As a measure for the amount of prostate fluid present in the urine sample and abnormal kidney function, urinary total PSA and creatinine were used for normalization. Both urinary proteins were measured in urine with Roche-developed assays for creatinine (CRE2U, ACN 8152) and PSA (total-PSA, 04491734 016) by the Erasmus MC Department of Clinical Chemistry using a Roche Cobas 8000 Modular Analyzer (Roche, Woerden, the Netherlands).

Statistical analyses

GraphPad Prism 6 was used to visualize results and for statistical analyses. Unpaired T-tests were used to calculate p-values of expression values between different patient groups. Furthermore, this software was used to calculate correlation coefficients (r^2) and ROC-curves.

RESULTS

For optimization of the assay, several experiments were conducted. First, capturing exosomes via biotinylated antibodies to a streptavidin-coated plate versus direct exosome coating to a MaxiSorp 96-well plate was compared. Using biotinylated antibodies on streptavidin-coated plates increased signal intensity and lowered background signals (Figure 1A). Second, unconjugated CD9 or CD63 antibodies were directly coated on MaxiSorp plates. This resulted in up to 3 times higher background signals and limited signal increase when an increasing amount of exosomal proteins was added (Figure 1B). Third, we tested whether the detergent/emulsifier Tween-20 could reduce background TR-FIA signals. Despite the theory that detergent might dissolve exosomal lipid membranes and impair the TR-FIA exosome detection, washing with Tween-20 0.05% improved Europium signal intensity (Figure 1C). Fourth, as a negative control for Europium labeling, exosome-specificity was tested using Europium-labeled KLK2 which is not present on exosomal membranes. As expected, no signals were observed above background (Figure 1D). Fifth, FCS is rich in proteins and bovine-derived exosomes and therefore could interfere with the capture and detection of cell-derived exosomes. We spiked purified VCaP exosome samples (1 µg protein) in culture medium containing different concentrations (0-40%) of FCS. We observed no statistical difference, indicating that our assay does not detect exosomes in FCS and that FCS does not affect human-specific detection of vesicles (Figure 1E).

The conditions we chose for the capture and detection of exosomes included a sandwich assay with streptavidin coated plates, biotinylated CD9/CD63 antibodies and Europium-labelled CD9/CD63 antibodies. Tween 20, at a concentration of 0.05%, was added to wash buffers to reduce background and increase CD9/CD63 signals.

Sensitivity analysis of the TR-FIA with purified exosomes

After optimization of the TR-FIA, sensitivity was tested with purified exosomes from the cell lines PC346C and VCaP. A minimum of 9.39 ng exosomes per well in 100 µL (measured by amount of protein present in purified exosome preparation) was enough to reliably measure CD9 signal. Capturing with biotinylated CD9 antibody and detection with the Europium-labeled CD9 antibody (CD9-assay) showed highest sensitivity for these samples after background correction (Figure 2). For capture with biotinylated CD63 antibody and detection with Europium-labeled CD63 antibody (CD63-assay), the lowest measurable amount of protein was 18.75 ng with VCaP exosomes and 37.5 ng with PC346C exosomes (Figure 2).

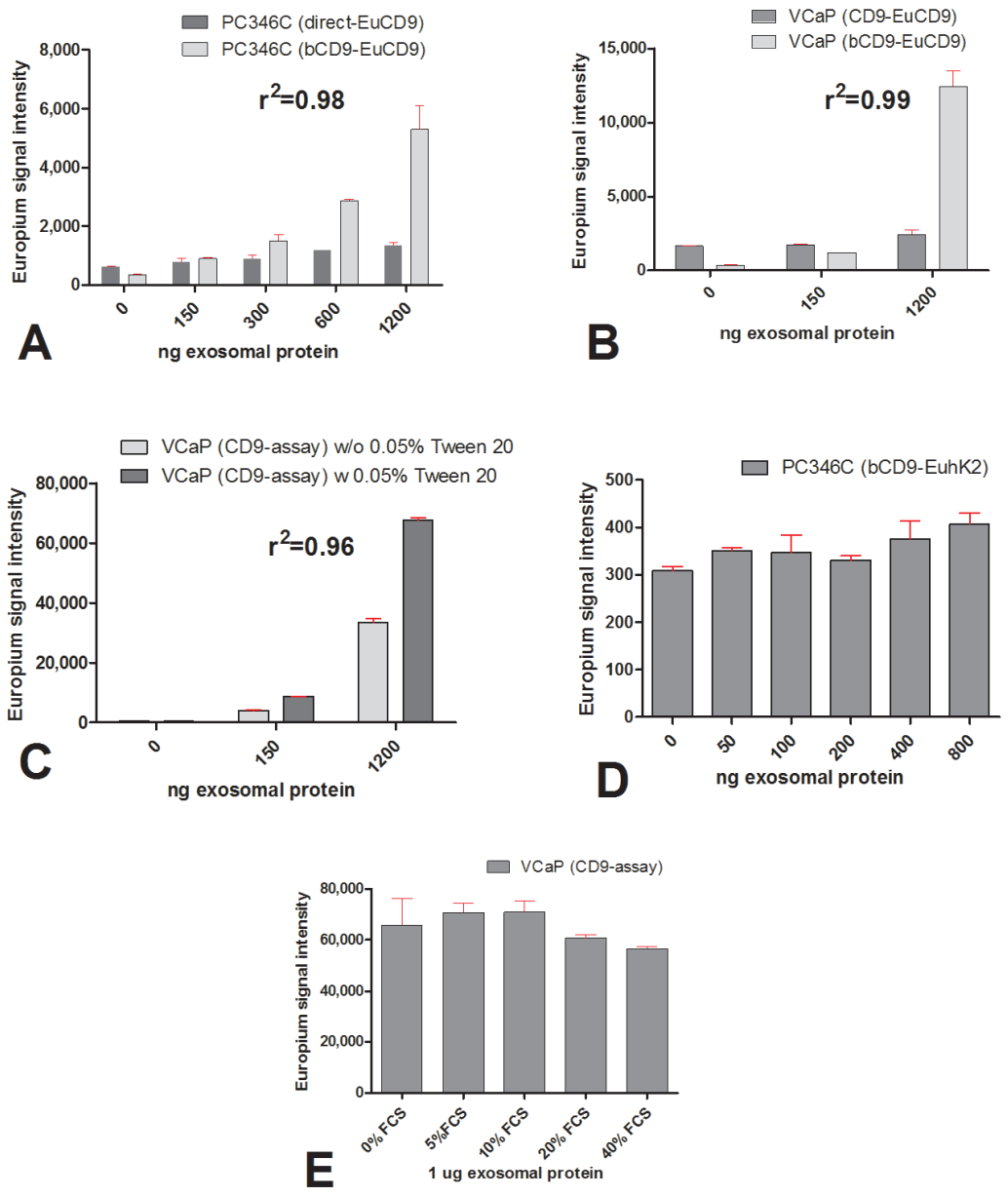

Figure 1. Development of the TR-FIA (data on the CD63-assay not shown). (A) direct application of PC346C exosomes to uncoated plates without the capture antibody (direct) versus using streptavidin-coated plates with the biotinylated capture antibody (bCD9). Both exosomes were measured with europium-labelled CD9 antibody. (B) MaxiSorp plates were coated with non-biotinylated CD9 antibodies and compared with streptavidin plates that were coated with biotinylated capture antibody (bCD9). Both exosomes were measured with europium-labelled CD9. (C) Addition of 0.05% tween 20 in the wash buffer was tested in the CD9-assay. (D) Application of a Europium-labelled KLK2 in our assay as a control for membrane-specific binding. KLK2 is a protein secreted in a different cellular pathway and normally not present in exosomal membranes. (E) We spiked purified VCaP exosome samples (1 µg exosomal protein) in culture medium with 0-40% FCS and performed the CD9 TR-FIA. All measurements were performed in triplicate and variance was estimated with the standard error of the mean (SEM).

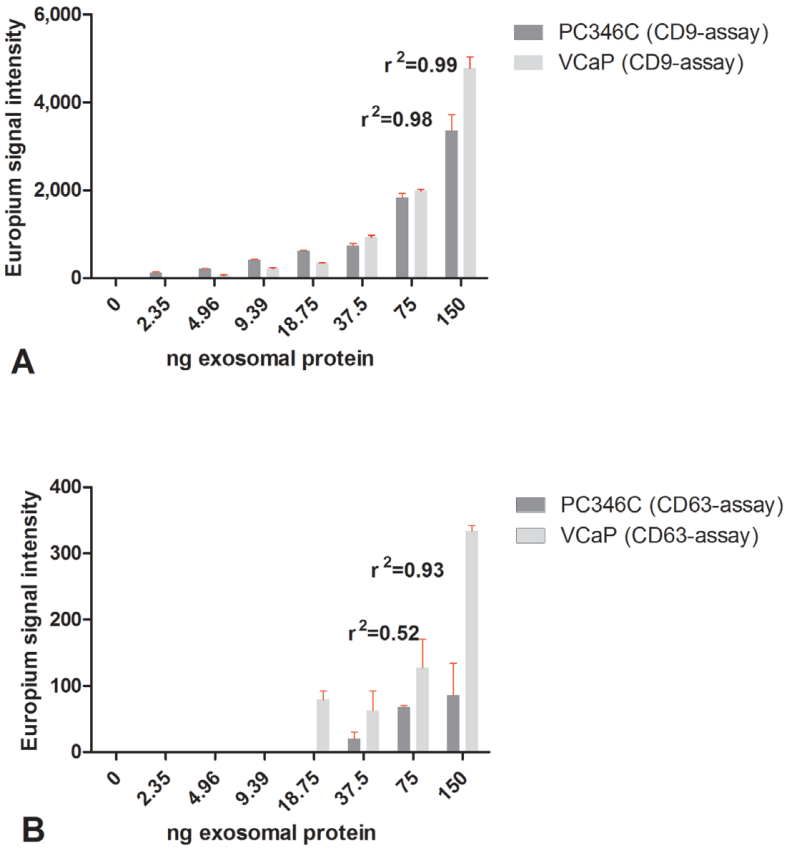

Figure 2. CD9-assay (A) and CD63-assay (B) on purified exosomes from the PC346C and VCaP cell culture medium. The amount of protein in purified exosome preparation is used as a measure for the number of exosomes (ng exosomal protein). All measurements were performed in triplicate and variance was estimated with the standard error of the mean (SEM).

TR-FIA with cell culture medium

The next step was to test whether minimally processed (only centrifuged once at 3000 x g) cell culture medium and urine samples could directly be used for TR-FIA analysis. Serum free cell culture medium of the LNCaP cell line was collected after 48 h exposure to cells (when 80-100% confluency was reached) and tested in the CD9 and CD63 TR-FIAs (Figure 3). High Europium signals were found within 100 µL cell culture medium and dilution series revealed a high linear correlation. After dilution of LNCaP cell culture medium, samples were aliquoted and used as calibration curve in all following experiments. Subsequently, we tested 100 µL cell culture medium from 37 cell lines (Figure 4). In almost all cell lines both the CD9 and CD63 assay showed a signal above background, but levels varied dramatically. Some human cell lines had extremely low signals for CD9 but were positive for CD63 (e.g. PNT2C2, TOV21G, H460, H295R and U2OS2-G3) or vice versa (e.g. EVSA-T, MOA MB453M and BPH1). Overall, the correlation between CD9 and CD63 signals among these cell lines was weak (r2=0.1816).

FCS (bovine origin), which is often supplemented in cell culture medium, showed no signals in the assay (Figure 4, control medium). Also, no or very low signal was measured in non-human (mouse and dog) cell line medium. These findings are in agreement with the antibody specificity against human CD9 and human CD63.

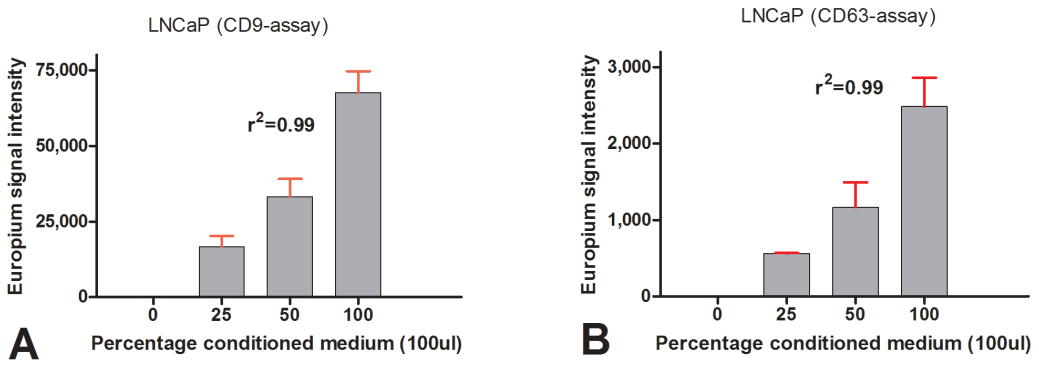

Figure 3. Dilution-assays with CD9 (A) and CD63 (B) of LNCaP cell culture medium. 100 µL cell culture medium was two-fold diluted for 4 times. As a control (0) medium was taken that was not exposed to cells. This control was used to correct for background signals. All measurements were performed in triplicate and variance was estimated with the standard error of the mean (SEM).

TR-FIA with urine samples

A total of 135 patient urine samples (100 µL per well in triplicate) were analyzed with the CD9 and CD63 TR-FIA. Urine that was collected after DRE showed significantly higher Europium signals (Figure 5). Urine from men without a DRE or treated by radical prostatectomy did not significantly differ in CD9 and CD63 levels. Women had the lowest signals compared to any other group tested. Figure 5 also depicts Europium counts between the samples (after DRE) with or without PCa in the two different assays. No significant difference was found between men with PCa and without PCa (CD9 p=0.166, CD63 p=0.223).

Since variability in DRE and urine volume will result in fluctuating concentrations of prostate (cancer) fluid in the voided urine, a correction factor is needed. We have chosen the urinary prostate-specific antigen PSA (UPSA) as a measure for the contribution of prostate fluid in the urine. In addition, urinary creatinine (UCr) was measured in each sample to analyze potential effects of differences in renal function. Distribution of UPSA (mean 3446.2, range 0.16-57100) and UCr (mean 10.4, range 1.7-30.2) is shown in supplemental Figure 1. No significant difference was observed in UPSA and UCr between men with and without PCa. UCr was lower in women, which is well known (data not shown).[20] As expected, UPSA was much higher in urine from men that underwent DRE (on average 6171.3 ng/ml) and significantly lower in urine from men without DRE

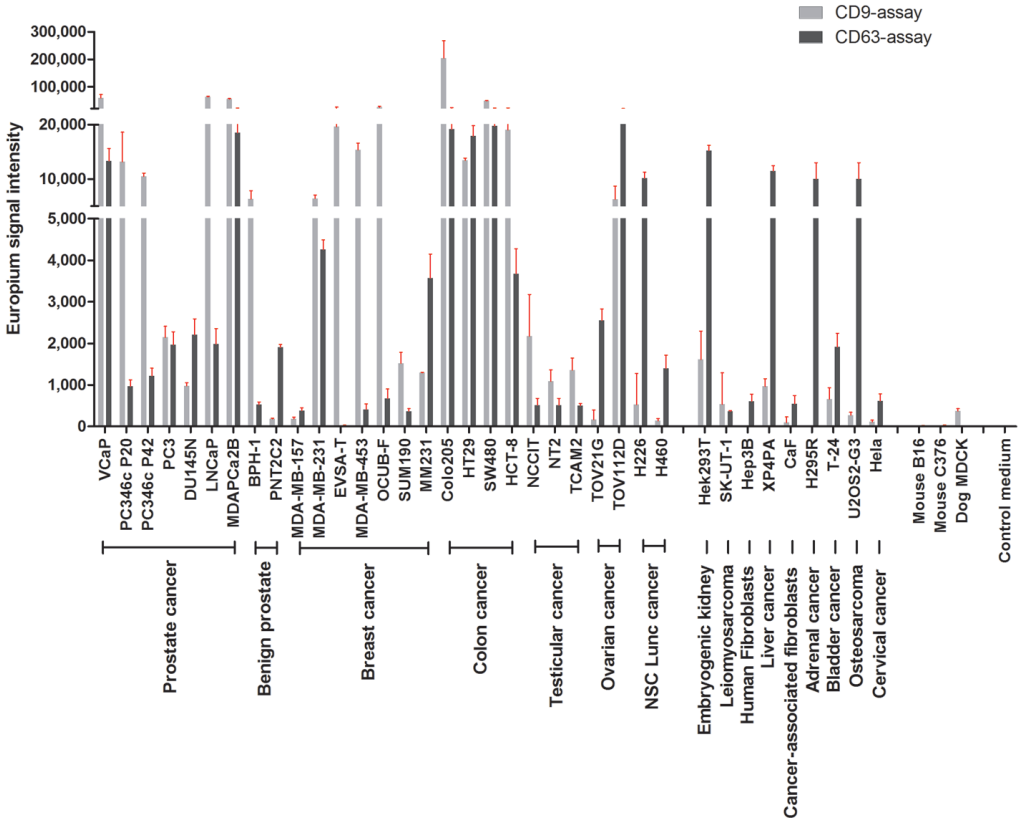

Figure 4. Cell culture medium from cell lines (n=37) from different human malignancies was tested in the CD9 and CD63-TR-FIA. Two mouse cell lines and one dog cell line were taken as control. Cell lines were cultured up to 80-100% confluency. When this was reached, medium was taken and centrifuged at 3000 x g to remove cellular debris. Measurements were performed twice with 100 µL independently from each other. Individual signal intensities were corrected for background (unexposed medium) and differences between measurements was corrected based on the same LNCaP control concentration series that was applied in both measurement. All measurements were performed in triplicate and variance was estimated with the standard error of the mean (SEM).

(on average 317.2 ng/ml (p=0.032). Men treated by radical prostatectomy and women mostly had undetectable UPSA levels (data not shown).

After normalization for UCr the CD9 and CD63 assay showed no significant difference (Figure 6) between urine from men with and without PCa (both after DRE). However, when normalized for UPSA, the CD9 and CD63 signals were significantly higher in urine from PCa patients (p=0.0006; p<0.0001). The mean of the Eu-signal/UPSA ratio increased from 6.6 to 18.1 for CD9, whereas the mean ratio for CD63 increased from 16.1 to 58.2. The Receiver operating characteristic (ROC) curve for the CD63 assays showed an Area under curve (AUC) of 0.68 (Figure 7), indicating that within this small cohort, the TR-FIA has higher diagnostic accuracy in detecting prostate cancer than CD9 (AUC = 0.58) and serum PSA (AUC = 0.61).

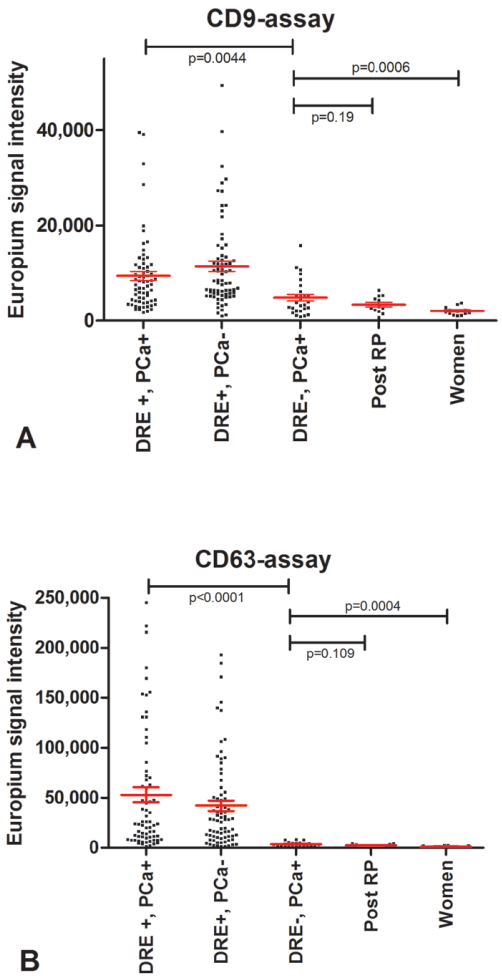

Figure 5. CD9-assay (A) and CD63-assay (B) on urine samples after DRE (DRE+) from men with (n=67) or without PCa (n=76), men with PCa (n=16) without DRE (DRE-), patients after radical prostatectomy (n=13) and women (n=16). Average of triplicate measurements is shown. All measurements were performed in triplicate and variance was estimated with the standard error of the mean (SEM).

DISCUSSION

The goal of this study was to design a non-invasive, sensitive assay for detection of (prostate cancer) exosomes in body fluids, which could be easily implemented in a clinical setting. We have reached most of these aims by constructing a TR-FIA using streptavidin-coated plates, biotinylated capture antibodies and Europium-labeled detection antibodies. This TR-FIA enabled us to detect prostate-derived vesicles with high sensitivity and over a broad dynamic range in shortly centrifuged (at low speed) post-DRE urine.

Since we use the same monoclonal antibody for capture and detection we do not measure single CD9 or CD63 molecules but distinct molecules kept together in membrane structures of, for example vesicles. Shortly centrifuged urine supernatant will not only contain the 30-150 nm (diameter) size exosomes, but also larger extracellular vesicles

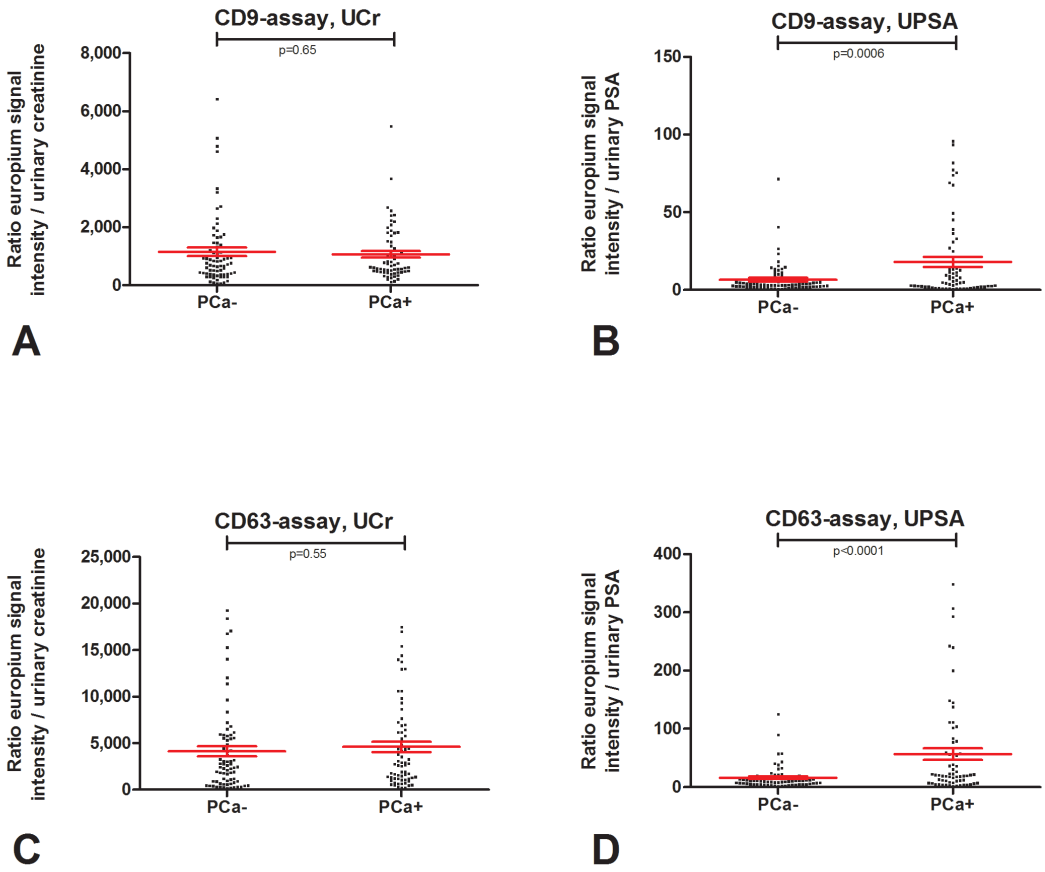

Figure 6. CD9-assay (A and B) and CD63-assay (C and D) on urine samples from men with (n=67) or without PCa (n=76), after DRE. Assays were corrected for urinary creatinine (UCr. A and C) or urinary PSA (UPSA, B and D). All measurements were performed in triplicate and variance was estimated with the standard error of the mean (SEM).

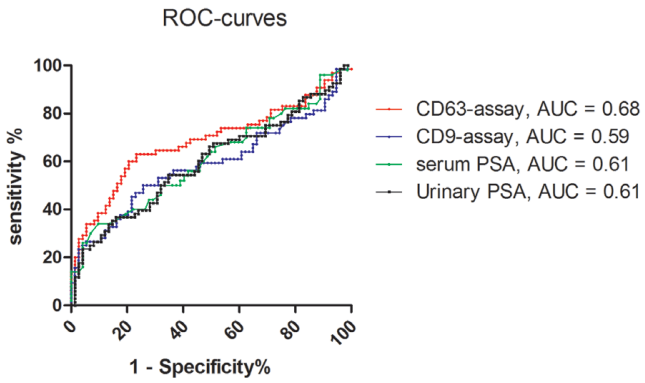

Figure 7. A Receiver-operating curve (ROC) for the CD63 and CD9-assay after correction for urinary PSA (UPSA). Serum PSA and urinary PSA from patients were also analyzed with a ROC.

and membrane debris.[21,22] As long as multiple CD9 or CD63 molecules are present, any membrane fragment can give a positive TR-FIA signal.

The TR-FIA signal measured is determined by the number of exosomes present, the number of antigens per vesicle and the affinity of the antibody to the epitope. For example, the high CD9 and CD63 signals in medium from COLO205 could mean that many exosomes were secreted and/or a high concentration of the antigens on each exosome. With the currently available techniques for quantifying exosomes such as flow cytometry, nanoparticle tracking analysis and tunable resistive pulse sensing, it is possible to estimate the number of vesicles. Unfortunately these techniques still have many restrictions such as vesicle size detection limits, quantitation accuracy and laborious procedures for regular and high throughput use.[6] Whether these technologies correlate to the TR-FIA outcome, needs to be established. Regarding the clinical use of the TR-FIA for detection of prostate cancer, the difficulty to quantify vesicles is not necessarily a limiting factor as long as the signal is reliably measured and significant for the presence of disease. A complicating factor that arises within our TR-FIA and could influence results is the presence of viruses that use outward-budding from host cell membrane to form their envelope.[23] For the HIV-1 virus it is known that this envelope contain the transmembrane proteins CD9 and CD63.[24] We used exosomes from the VCaP and PC346C cell line that were provided from xenografts, which produce XMRV-like viruses. If these viruses also produce envelopes containing CD9 and CD63 is unknown, but if this is the case it could produce a signal in the TR-FIA.

In order to be able to check and correct for variability between TR-FIA plates, we used the high CD9 and somewhat lower CD63 signals measured in LNCaP cell culture medium. A large batch of medium was prepared and stored and new aliquots were used for each experiment. In the future, a more robust common universal standard, such as synthetic double-epitope peptides, will have to be devised for assay calibration.

One of the major improvements of the described assay over currently available conventional ELISAs is the use of Europium chelate used as a label for time resolved fluorescence.[25] The main advantage of Europium is that the fluorescence emitted after excitation is long lived as compared to auto-fluorescence. Therefore, this technique reduces background and enables us to measure with high sensitivity. Furthermore, Europium can be measured over a much broader dynamic range (signal intensity 50 – 300,000) as compared to conventional ELISAs (e.g. HRP-based). Other strategies to improve immunoassays include the adapted proximity ligation assay (PLA) and amplified luminescent proximity homogeneous assay using photosensitizer-bead.[26,27] Also these technologies show high sensitivity and the ability to measure vesicles in low volumes of bodily fluids but are relatively more labor intensive.

CD9 and CD63 are transmembrane proteins that are 7-10 times enriched in exosomes and are used as a general marker for exosomes.[28,29] CD9 was shown to be higher ex-

pressed in exosomes from prostate cancer, but also in other types of malignancies. Furthermore, CD9 was reported to have a specific role in metastasis.[4,30-32] We showed that CD9 and CD63 are present on exosomes from almost all cell lines with varying signal intensities. Although CD9 and CD63 are considered common extracellular vesicle markers, we showed that these markers are detectable on exosomes from almost all cell lines (n=37) but with highly varying expression levels.

Control medium with FCS and animal cell lines such as those derived from mouse and dog showed no or very low signals. This demonstrates that our assay based on antibodies against human CD9 and CD63 is indeed human-specific. The spike-in experiments revealed that high concentrations of FCS do not interfere in the detection of markers in the TR-FIA.

Although CD9 and CD63 are not prostate (cancer)-specific, we could use their presence as a marker for prostate-derived exosomes in urine, since levels were very low in urine from women, men after prostatectomy and men before DRE. This indicates that the number of exosomes and/or the levels of CD9 and CD63 on exosomes from kidney and bladder are low in urine as compared to prostate-derived exosomes after DRE. This is particularly evident for CD63. Initially, no statistical difference was observed in CD9 and CD63 signals between men with and without PCa. Urinary creatinine, a marker for kidney function showed no additional value as a correction factor in our assay. Although serum PSA can be elevated in prostate cancer, urinary PSA itself has shown not to be different between healthy men and men with prostate cancer and therefore could function as correction factor for DRE in our assay.[33-36] After correction for the relative amount of prostatic fluid after DRE using UPSA, a statistically significant difference was observed for both markers. These observations show that a DRE is currently an essential element of the urine collection. One could argue that a DRE is not necessary anymore if the CD9 and CD63-assays would be more sensitive. However, as a consequence of not performing a DRE, the lower ratio of prostate (cancer) exosomes among other urinary exosomes (from bladder and kidney) might result in loss of distinction between men with and without PCa. This issue could be resolved by using antibodies that specifically recognize prostate (cancer)-derived exosomes. Antibodies against known prostate (cancer)-specific transmembrane proteins need to be tested in the TR-FIA. Exosomal transmembrane proteins have been identified using mass spectrometry, but so far few of these proteins have been found higher expressed in cancer and none to be cancer-specific.[4]

The assay we developed reveals that measurements of urinary vesicles can indicate the presence of PCa. Because we only measured two markers in urine of men with or without PCa, it is unclear whether this assay is affected in men with other diseases of the urinary tract and in particular in diseases of the prostate such as benign prostate hyperplasia or prostatitis. Other steps to take include determining the assay robustness and intra-person variability at different time-points. In addition, we will further have to

select the optimal capture and detection markers as discussed above. The CD63 TR-FIA has an AUC of 0.68 and although this is already better than serum PSA alone in our cohort, it still does not fully address the clinical needs.

In this study we only assessed this assay as a diagnostic test. The limitations of the PSA assay results in taking unnecessary biopsies in approximately 68% of men with PSA higher than 3 ng/mL. In addition, with the cutoff of 3 ng/mL, approximately 13% of prostate cancers are missed.[37] In our cohort, the CD63 TR-FIA outperforms the PSA assay in diagnosing prostate cancer. Logistic regression analysis showed no independence between the two assays ($p>0.05$). Even more relevant would be to predict whether the identified PCa should be treated or is insignificant and active surveillance is a valid strategy to follow the patient. The main marker for prognosis currently is Gleason score. Since our cohort is based on men entering our clinic for their first consult, there is a strong bias towards Gleason score 6. In order to determine whether the TR-FIA has diagnostic and prognostic value, a larger cohort balanced for different Gleason scores needs to be analyzed. In conclusion, the CD63 TR-FIA could influence patient management with respect to making decisions on taking biopsies, while a role in tracking patients on active surveillance and therapy selection is still to be investigated.

CONCLUSION

The presented TR-FIA enabled us to measure transmembrane proteins on vesicles directly in urine and cell culture medium with low background signals and high sensitivity. CD9 and CD63 are exosomal markers that show higher signals in men with PCa after DRE and correction for urinary PSA. More antibodies need to be tested using this TR-FIA to discover the most optimal combination of diagnostic and prognostic PCa markers.

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SUPPLEMENTARY DATA

Supplementary data for this article are available online at:
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PART 3

General discussion and appendices

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Prostate cancer (PCa) is a heterogeneous disease with a variety in clinical, morphological and molecular genetic presentations between patients, within a patient, intertumoral and intratumoral.[1,2] This heterogeneity causes many different PCa phenotypes with multiple clinical outcomes. Over the past decades clinical and molecular researchers have begun to unravel these different subclasses of PCa for more accurate diagnosis and a better estimation of prognosis.[3] With this information, clinicians are increasingly able to select the most optimal individual treatment options (personal medicine).[4]

Current markers that enable us to determine diagnosis and prognosis in PCa, such as prostate-specific antigen (PSA), have shown to be a useful marker for daily clinical use.[5] Unfortunately, PSA lacks specificity to distinguish between low risk PCa, high risk PCa and benign prostatic diseases. Consequently, the use of PSA with a specific cut-off value has shown to be related to a high risk of overdiagnosis and overtreatment.[6] Novel biomarkers have to be found for better diagnosis and more reliable prognosis.

Despite rapid advances in technology, few biomarkers have made it to (pre-) clinical implementation.[7] One of the problems with biomarker research is the so-called dynamic range problem. Especially regarding mass spectrometry, few high abundant proteins (e.g. albumin, immunoglobulins) overshadow low abundant proteins. The most interesting candidate markers are probably among these low abundant serum proteins (concentration of 10^{-3} ng/mL to 10^{-5} ng/mL).[8] Even with current "state-of-the-art" technologies, discovering novel biomarkers remains challenging.[9] As stated in the objectives of this thesis, we aimed to identify novel biomarkers for PCa that could help us distinguish between normal prostate tissue and PCa, but also estimate prognosis more accurately.

DISCOVERY OF BIOMARKERS WITH EXTRACELLULAR VESICLES

Since the beginning of this millennium, small tissue-derived extracellular vesicles (EVs), often referred to as exosomes, have been shown to be present in seminal fluid, urine (with or without DRE/prostate massage) and serum. Because of their biosynthesis and excretion pathway, they contain a wide range of proteins and RNA that represent their tissue of origin.[10-12] Many different types of vesicles have been described with their own distinct characteristics (e.g. content, size and origin). These differences in nomenclature lead to confusion and made comparing vesicle research difficult. In order to improve collaboration between researchers, the International Society of Extracellular Vesicles (ISEV) was officially founded in the beginning of 2012.[13] Involving all members of the society it was decided that the collective name of any type of vesicle is 'extracecullar vesicle' (EV). Also, all data from profiling EVs was combined to improve discovery and has led

to an extensive online database such as ExoCarta with more than 286 studies included, discovering 9769 unique proteins, 3408 mRNAs and 2838 miRNAs.[14] Other databases that have been compiled and used are EVpedia, VesiclePedia and exRNA.[15-17]

The advantage of using these EVs for biomarker discovery is our ability to purify them from complex biofluids and therefore profiling their content is less hampered by high abundant proteins that are present in serum, plasma or urine. Furthermore, these vesicles express specific transmembrane proteins that could be used for more specific isolation and detection. In 2011 we published an overview of research regarding different aspects of biomarker discovery for PCa by using EVs.[9] Together with our own efforts, in the intervening seven years progress has been made and novel candidate markers have been proposed.

Diagnosis

Using MS-MS we identified 866 proteins, from which 263 proteins were differentially expressed between EVs from cancerous and non-cancerous prostate cell lines.[18] From those proteins, 10 were significantly higher expressed in the PCa cell lines. We selected PDCD6IP, FASN and XPO1 as most promising candidate novel EV biomarkers and validated their high expression. When we compared our complete list of differentially expressed proteins and with Sandvig *et al*.[19] and Hosseini-Beheshti *et al*.[20] only 9 showed overlap, where only our candidate marker PDCD6IP was also identified by Sandvig *et al*. Unfortunately, our study was the only one where PDCD6IP was higher in PCa-derived EVs. All 9 overlapping proteins where already shown to be identified in many EVs, also from non-PCa cells. Sandvig *et al*. showed CDCP1 and CD151 as candidate protein markers, whereas Hosseini-Beheshti *et al* found ANXA2, CLSTN1, FLNC, PSMA and GDF15 to be higher expressed in PCa. CLDN3 and GGT were also identified as candidate markers.[21,22] Further in depth proteomic analysis of EVs from clinical samples (prostatic secretions in urine) showed PSA, ACPP, TGM4 and PSMA to be higher expressed.[23] In urine TMEM256, ADIRF, PCYOX1 and LAMTOR1 showed highest correlation with PCa.[24] With an immune-based assay, CD9 and CD63 were shown to be able to differentiate between PCa and a benign prostate by applying 100ul unprocessed urine.[25] The clinical impact and variety of markers is discussed below.

Regarding RNA, PCA3, ERG and the TMPRSS2:ERG fusion gene were also found to be higher expressed in EVs in urine from PCa patients.[26-29] In urine from PCa patients miR-21, miR-141, miR-375, miR-483-5p, miR-1275, miR-1290, miR-107 and miR-574-3p were shown to be higher expressed.[30-32] miR-196a-5p and miR-501-3p were downregulated in urinary EVs from PCa patients.[33] These findings suggest that microRNA from EVs might serve as a marker for PCa.[34] These markers need to further studied in large patient cohorts to elucidate their true potential.

Many studies profiled EV-content and revealed promising candidate markers for PCa. Despite more tissue/disease specific selection of these proteins/miRNA by using EVs and therefore bypassing the dynamic range problem, published studies show no overlap in their most distinctive markers. One of the explanations could be the variety of techniques used for identification of protein/RNA markers used among the different studies. Especially in mass spectrometry, quality and resolution (better accuracy mass-to-charge ratio) have been improved over the years and tandem mass spectrometry (MS/MS) for *de novo* protein sequence information was introduced.[35,36] A second explanation could be that even with these current techniques, there are still too many high abundant (most likely less interesting) proteins in EVs. Thirdly, the use of stringent cut-off values for selection of most differential expressed proteins could be part of this problem. Every study tries to select 3-5 most promising candidates, whereas there probably is more overlap when less stringent cut-off values were applied (e.g. top 20). A fourth possible problem that could contribute to these differences, is the use of different cell lines and patient groups. Even between PCa cell lines there is a difference in specific protein expression.[25] This difference is probably also present between or within patient groups and subsequently could influence identification. Fifth, isolation techniques for EVs and their content could introduce variations in concentration and purity.[37] Especially in PCa, rectal massage or digital rectal exam (DRE) causes more prostatic fluid in the urethra/urine and major alteration in protein identification.[29] In our study (Chapter 7), we showed an enormous increase in the number of urinary EVs upon DRE.[25] It is expected that PCa urinary PCa markers are much more abundant after DRE and therefore remains important for future assays.

With improving techniques and increasing sensitivity we should keep on searching for new markers (protein and RNA) and profile more samples from well characterized patient groups. Besides identification of a single marker, future research should also focus on a panel of markers that could possibly better predict significant disease and more reliable prognosis. Some clinically available tests that already use such a combination to predict the chance of high risk PCa prior to biopsy (Table 1).

Regarding our own work and the identification of XPO1 as most promising candidate marker for PCa, it would be interesting to test if this protein has any clinical relevance in other diagnostic tests besides Gleason score, surgical margins and pT stage. Besides IHC on samples after invasive biopsies or radical prostatectomy, currently no non-invasive diagnostic tests are available for direct measuring XPO1 in serum or urine, therefore direct translation to the clinic remains difficult. It would be interesting to proceed with the exploration of the clinical value of XPO1 and subsequently establish a reliable assay for this marker.

Table 1. Overview of commercially available blood/urine assays for PCa based on a panel of markers that are applicable prior to prostate biopsy.

Test	Source	Substrate	Clinical relevance	Reliability
SelectMDx[38]	Urine (after DRE)	mRNA DLX1 and HOXC6 vs KLK3	Probability for high risk PCa	AUC 0.87
ExoDx Prostate (Intelliscore)[39]	Urine	EV-derived mRNA PCA3, ERG and SPDEF	Probability for high risk PCa	AUC 0.74
Michigan Prostate Score (MiPS)[40]	Urine (after DRE) and blood	mRNA TMPRSS2:ERG and PCA3 and serum PSA	Probability for high risk PCa	AUC 0.73
Prostate health index (PHI)[41]	Blood	Total PSA, free PSA and [-2] proPSA	Probability for high risk PCa	AUC 0.72
4K score[42]	Blood	Total PSA, free PSA, intact PSA and human kallikrein-related peptidase	Probability for high risk PCa + risk of distant metastases within 20 years	AUC 0.80

Prognosis

Besides markers for diagnostic purposes, it is interesting to know if EV markers could be used to determine prognosis to prevent invasive treatment such as radical prostatectomy for insignificant PCa. Also, it is interesting if these markers can be used to predict response to therapy. Unfortunately, the number of reports on prognostic markers is limited. We tried to correlate XPO1-expression to several clinic-pathological parameters but did not identify such a correlation. Other studies showed substantial clinical relevance regarding decreased expression of miR-34a in PCa progression and poor prognosis *in-vitro*.[38] In clinical plasma samples miR-1290 and miR-375 were shown to correlate with poor survival in castration resistant PCa (CRPC).[39] High expression of miR-141 and miR-375 in plasma was found in patients with metastatic PCa.[31] One study found miR-2909 to be higher expressed in urine from patients with high risk PCa.[40] Yu *et al*. profiled miRNA in serum EVs from a small group PCa patients before they started with radiotherapy.[41] They found a set of miRNAs that could predict therapeutic effect. An *in-vitro* study by Kharaziha *et al*., showed that MDR-1, MDR-3, Endophilin-A2 and PABP4 to be enriched in the docetaxel resistant DU145 cell line.[42] Within the docetaxel resistant PC3 cell line, but also in clinical samples, P-glycoprotein (P-gp) was higher expressed.[43] Interestingly, when P-gp was knocked down, the sensitivity to docetaxel increased. Kawakami *et al*. showed ITGB4 and VCL were upregulated in docetaxel resistant PC3 cell line. Silencing of these proteins showed no alteration in proliferation and Taxane resistance but showed attenuated cell migration and reduced invasion.[44]

Recently, the AR-V7 mRNA was identified as a predictive marker for response to the anti-androgen enzalutamide and the CYP17 inhibitor/anti-androgen abiraterone.[45] The primary discovery was made using circulating tumor cells, but Del Re *et al.,* showed that also plasma EVs contain the AR-V7 splice variant and can also be used a predictive biomarker. [46]

VALIDATION OF BIOMARKERS FROM EVS

Validation is an essential step in the process of biomarker assay development. This phase verifies the differential expression between samples and gives the opportunity to test the candidate marker in an independent validation set (patient cohort). Especially with *in vitro* studies, discovery of potential candidate markers is mostly validated with labour intensive techniques (e.g. Western blotting) and with the similar and limited number of EV samples. So far, there are very few studies describing the validation of EV-derived markers in large independent (patient) cohorts with enough power. Worst *et al*. validated the presence of CLDN3 in serum in 84 patients with a significant higher expression in localized high risk PCa (Gleason score ≥ 8).[21] Wang *et al*. showed that their mass spectrometry-identified markers also had higher expression in an immunoaffinity-based assay with urinary EVs from PCa patients (n=16).[47] Li *et al*. showed that miR-141 was higher in EVs isolate with ExoQuick in patients with PCa (n=20) and even higher in metastatic PCa.[48] Our group has shown that EV-derived markers (XPO1) could be validated with a tissue micro-array in a large group of patients (n=481). One urine EV-based assay made it into a clinical setting (ExosomeDx Intelliscore). This assay isolates EVs from whole urine (non-DRE) and measures the ERG and PCA3 mRNA expression as compared to SPDEF. Besides proof of differentiation between groups, it needs to be shown that the marker has independent added clinical value. The markers must add to established markers (e.g. Gleason score and PSA) or be cheaper or more convenient when as good as current practice.

In general, the number of reports that describe the validation and added clinical value of candidate markers in patient cohorts are remarkably low. The studies that are published use relatively small groups and labour-intensive techniques (isolation of EVs via ultracentrifugation or ExoQuick) that are unsuitable for daily practice or high-throughput analysis. In order to make a validation assay, it is important to make it reproducible, easy to perform and with the possibility to analyse many samples on one platform. Also, when analysing EVs from bodily fluids (e.g. serum, urine or semen) it is important to choose which material to utilize. The most ideal material has to be taken via minimally-invasive techniques. Because discovery of biomarkers from serum is hampered by the abundance of many analytes, urine is an interesting and slightly less complex source.[49]

TECHNIQUES FOR ENRICHMENT AND CHARACTERISATION OF EVS

Isolation of EVs is classically performed by ultracentrifugation. This technique has been well developed and can be used to process up to 250 mL of a single sample.[9]

Unfortunately ultracentrifugation requires expensive equipment and is time consuming (>5 hours) and cannot be performed high throughput. In order to reduce the time for isolation, multiple techniques have been developed such as ultrafiltration, precipitation, affinity capture and size exclusion chromatography. Although less laborious, each one of these techniques have issues with yield, purity, costs and/or isolation of EV subpopulations.[50]

Furthermore, we are currently not able to absolutely quantify EVs and analyse them on a single particle level.[25,51] Since the number of exosomes could possibly be useful for correction of assay input, but also have diagnostic or predictive value[52], quantification is an important step. Current techniques that are utilized, such as nanoparticle tracking analysis, tunable resistive pulse sensing and flow cytometry show promising results but have their own set of limitations.[51,53-56] Besides technical restrictions of quantification of EVs, a major challenge is the isolation and quantification of subsets of EVs, particular the cancer derived EVs because serum or urine contain a heterogeneous pool of EVs, derived from various tissues. Flow cytometry is capable of tissue-specific analysis of EVs in a complex fluid. An assay that can count or define EV subpopulations is typically based on immune-affinity. Antibodies directed against transmembrane proteins expressed on EVs (e.g. CD9, CD63, PSMA) can be used for tissue-specific isolation and characterization.[57] Previous reports have shown that EVs can be isolated from cell culture and plasma with an ELISA or with (magnetic) beads.[58-62] With our own efforts we were able to establish a reliable and highly sensitive TR-FIA (time-resolved fluorescence immunoassay) by using antibodies against the transmembrane proteins CD9 and CD63.[25] With this assay we have shown that EVs from urine from PCa patients had higher expression of these transmembrane proteins after correction for urinary PSA. Although showing correlation with PCa, these proteins are known as general markers for EVs.[63,64] Ideally more PCa-preferential transmembrane proteins, such as PSMA, need to be tested that might predominantly recognize PCa-derived EVs.[65] Immune-affinity isolation seem to be ideal for EV-research, but unfortunately as a separate assay it is also time-consuming and therefore less attractive for daily clinical use. An assay that highly selectively captures EVs from body fluids and directly characterizes or measures its content of interest, would be most ideal. So far, our developed TR-FIA sums up most of these needs and seems to be promising for future research.

EVS AS BIOMARKER TREASURE CHESTS IN LIQUID BIOPSIES

The concept of personalized medicine is considered a new epoch in cancer management, where for each patient, clinical decision support can be provided regarding individual treatment. The clinical application of personalized medicine in PCa is broad and

compromise early detection, diagnosis, prognosis, prediction of treatment response and disease progression.[66] An important aspect of this approach is that each patient needs to be stratified, according to several individual and cancer characteristics. Currently the most important factors for PCa besides easy acquirable PSA and clinical stage (by DRE) are Gleason score and signs of metastases (CT-scan, MRI, bone scan and/or PSMA-PET). Unfortunately, Gleason score can only be obtained via invasive biopsies (with risk of complications) and for evaluation of metastasis a time consuming and expensive technique has to be applied. Non-invasive techniques, such as liquid biopsies, could be applied more often with low chance of morbidity. Especially for PCa it also has the advantage of reflection of many tumor subclones, whereas biopsies only represent one specific tumor region.[67] The most promising body fluid components as PCa biomarker are circulating tumor cells (CTCs), ctDNA and EVs (Table 2). CTCs and ctDNA harbour the same potential as EVs in liquid biopsies and have been used to predict clinical stage and monitor the course of PCa.[68-70] Unfortunately, they tend to be only present in blood in advanced stages.[71] So far, CTCs are clinically not useful for localized disease. Soekmadji *et al*. showed that CD9 positive EVs are higher expressed in advanced metastatic PCa with detectable CTCs.[72] Interestingly, the androgen receptor (AR) splice variant 7 (AR-V7) can be detected in plasma EVs from CRPC patients and seems comparable to AR-V7 detection in CTCs.[45] Resistance to hormonal therapy could potentially be predicted.[46] Although EVs seem to be a promising source of biomarkers, new EV-based assays for PCa have to established and evaluated in order to fully elucidate their true potential as liquid biopsy.

Table 2. Most promising non-invasive source for PCa biomakers from body fluids (serum/urine)

	Protein	RNA	DNA	Advantages	Limitations
Circulating tumor cells (CTC)	+	+	+	Quantification and analysis of content (e.g. AR-V7) helps in predicting outcome and treatment respons	Detection of CTC is stage dependant, mainly in advanced stages
Cell-free tumor DNA	-	-	+	Abundant in plasma. Reveal genomic alterations, predict outcome and treatment response	Only present in advanced stages
Extracellular vesicles (EVs)	+	+	-/+	Present and detectable in all stages of prostate cancer. Can be found in urine.	Smaller than CTCs and therefore could have a subfraction of all cellular proteins/RNA

LIPIDOMICS

Most publications on biomarker discovery using EVs, focus on their intravesicular proteins or RNA and extravesicular (transmembrane) proteins. Based on their biogenesis they also contain a bi-lipid membrane reflecting (subdomains of) the membrane of the

cell from which they are derived. This lipid aspect of EVs has not obtained sufficient attention. Lipid composition has been measured by mass spectrometry, thin layer chromatography and gas liquid chromatography.[73,74] Several reports have been published describing lipid content and their enrichment factors from cells to EVs (2-3 times more cholesterol, glycosphingolipids, phosphatidylserine and sphingomyelin).[75] How this enrichment occurs remains relatively unknown. Only few studies described lipid analysis from urinary EVs from PCa,[76] but only one study compared this lipid content between patients compared to healthy individuals.[77] High expression of lactocylceramide occurred in PCa patient and phosphatidylserine in samples from healthy individuals. Heavily underexposed, characterization of EV lipids from more PCa patients with different clinical stages could contribute to finding new lipid-markers.

EVS AND TUMOR BIOLOGY AND THERAPEUTIC IMPLICATIONS

The biogenesis of EVs has been described previously.[9] How this process is organized and which factors influence this process is still not fully known. We do know that multiple factors play an important role in formation and secretion. Endosomal sorting complexes (ESCRT) and multiple Rab-proteins regulate this process. Excretion of EVs has important regulatory functions, such as discarding unnecessary content from cells (lysosomal degradation), but also in cell-cell communication. Especially this latter function could be of interest in tumor biology. EVs express many transmembrane proteins that interact with recipient cells. Malignant cells could theoretically influence their surrounding cells and subsequently change their microenvironment to their own advantage. So far, several *in vitro* studies have shown that EVs from malignant cells do alter their microenvironment (e.g. promote angiogenesis) and promote tumor progression.[78-82] Also EVs from metastatic site-derived cell lines are taken up more efficiently by benign cell lines and increased proliferation and migration.[83-85] Delivery of the proteins via EVs could even could contribute to PCa progression and induce neuro-endocrine differentiation.[86-88] Several studies suggested that EVs from malignant cells released in the tumor-bone interface, are involved in pathological regulation of bone cell formation in the metastatic site.[89-91] An important finding from the last few years, is the role of EVs in acquiring chemotherapeutic resistance during therapy.[92] Although more and more publications report the role of EVs in tumor biology, more research is needed to fully understand how they interfere with their microenvironment. Understanding this process could potentially lead to novel treatment strategies for malignant diseases.

Multiple studies have shown EV composition and biology have an effect on recipient cells. These findings gave rise to the hypothesis that EVs itself or alteration of EV biology

could be used a therapeutic option. To date, the number of reports on EV-mediated therapy in PCa is limited but is gently increasing.

Alteration of EV biogenesis

Because emerging evidence links the presence of circulating EVs to PCa progression, some studies tried to alter exosome production/secretion in order to treat malignant diseases. Manumycin-A, a natural microbial metabolite, was shown to inhibit EV biogenesis and secretion by CRPC cells, but not in normal prostate cells. Unfortunately, no effect was observed on cell growth.[93] In breast cancer a similar effect was observed.[94] From pre-clinical data it was shown that EVs from adipose tissue derived stromal cells and menstrual stem cells inhibit PCa growth and angiogenesis. Therefore, these EVs from these cells could be a novel therapeutic strategy in PCa.[95]

Alteration of EV immunogenicity

In vivo tracking studies have found that when EVs are administered systemically, most of them are taken up by macrophages and do not reach the organ or cells of interest.[96] In order to reduce immunogenicity and improve their therapeutic effect on recipient cells, they can be 'coated' with a ligand. Ohno *et al.* showed that EVs can efficiently be delivering miRNA to EGFR-expressing breast cancer cells by genetically altering donor cells to express the transmembrane domain of platelet derived growth factor receptor fused to the GE11 peptide.[97] A similar approach was used to express Lamp2b on dendritic EVs in therapy for Alzheimer's disease.[98] To avoid genetic manipulation, EVs can also be loaded with iron nano-particles and chemotherapeutics.[99] By using a magnet close to the recipient cells, these EVs could be manipulated by magnetic force and subsequently delivering their drug. Unfortunately, genetic modification and iron loading of EV-secreting cells remains challenging and time consuming. A more practical approach by Kooijmans *et al.* is the use of ligand conjugated polyethylene glycol (PEG) to decorate EVs after production.[100] With this PEG coating they improved cell specificity and prolonged circulation time, potentially improving drug delivery. This approach can also be used to fuse EVs with functionalized liposomes to create smart biosynthetic hybrid vectors.[101] Interestingly, this fusion approach could theoretically enable efficient EV loading of pharmaceutical drugs. Regarding PCa, no reports harbouring these techniques have been published.

EV-targeted therapy

EVs have been shown to have multiple roles in cancer by interacting with target cells and the tumor environment (e.g. creation of pre-metastatic niche). With these abilities EVs can also contribute to cancer metastasis.[102] An interesting approach in therapy for malignant diseases is therefore EV-targeted therapy. Nishida-Oaki *et al*. used anti-CD9 and

anti-CD63 antibodies to deplete EVs and achieved a significant reduction in metastasis to the lung, lymph nodes and thoracic cavity in mice.[103] This effect was also observed in pancreatic cancer.[104] These results demonstrated the concept of inhibition of EVs as prevention of metastasis and could therefore be beneficial for patients by achieving longer survival with less comorbidity.

Delivery of biological or pharmaceutical agents

Although the number of reports on this topic in PCa is also very limited, Johnson *et al*. showed that EVs could be loaded with pharmaceutical agents.[105]. Saari *et al*. showed that when EVs from PCa cell lines were loaded with Paclitaxel and subsequently administered to PCa cells, drug delivery into recipient cells was observed and subsequently had an effect of cells.[106] Another approach could be loading of EVs with small interfering RNA (siRNA) by co-incubation and electroporation.[107,108] These siRNAs are small RNA and can alter gene expression in cells and have a potential beneficial effect. Milk-derived and siRNA loaded EVs showed reduction of a target gene in hepatocellular carcinoma.[109] The number of reports on this method is increasing, but one major limitation of this method is its lack of efficiency and scalability for loading siRNA into EVs. More research is needed to clarify if this method is suitable for daily clinical use.

The design of future studies and therapeutics should acknowledge the existence and role of EVs, and seriously consider strategies for manipulating or circumventing their effects *in vivo*.

From the work presented in this thesis and published by the EV community, it is clear that vesicles are highly promising with respect to disease biomarkers and novel therapy interventions. The first EV-assays utilized in a clinical setting are on their way and many more are expected once robust assays are developed and the EV-markers independently validated.

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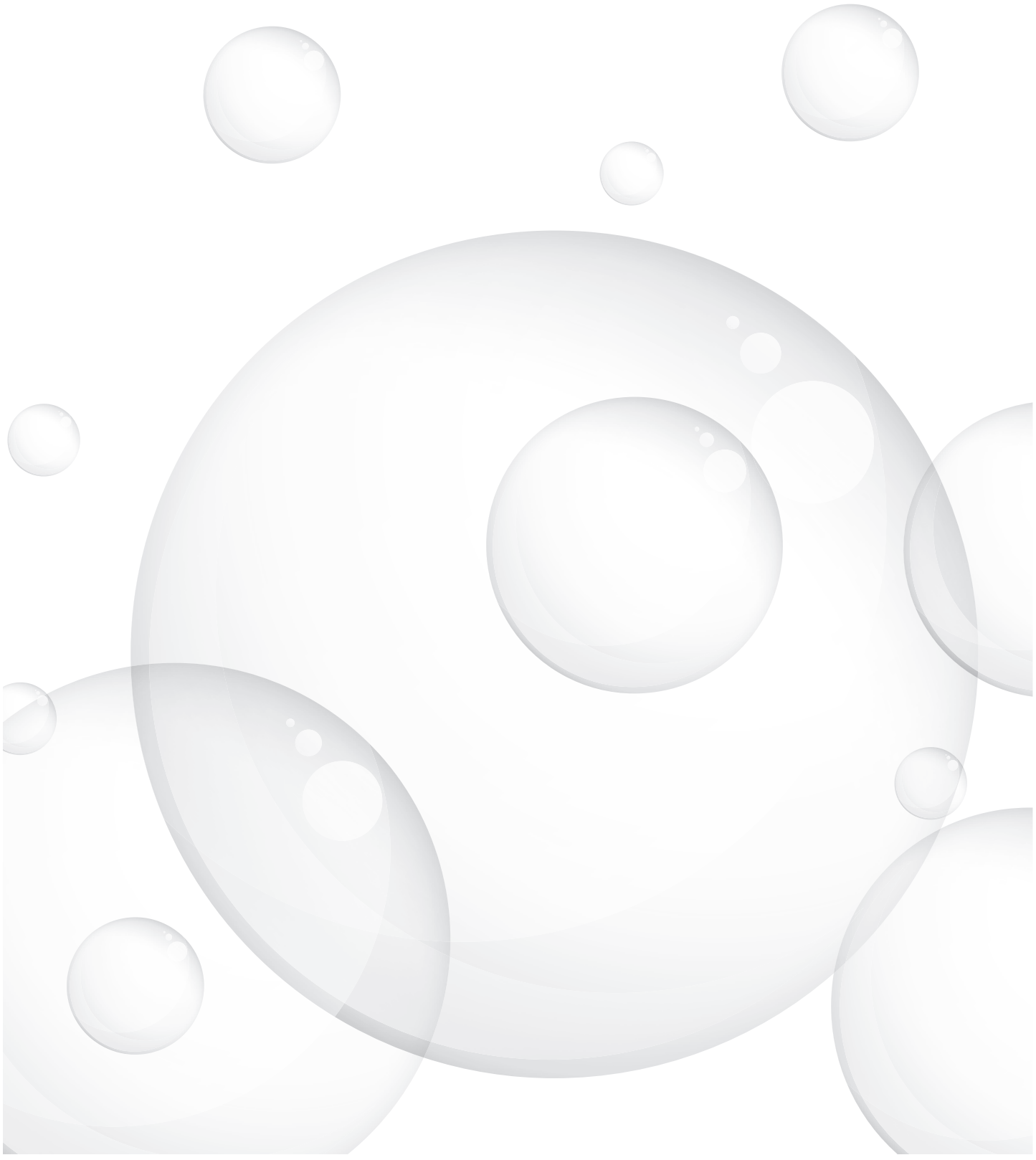


Summary

Prostate cancer (PCa) is the second most common malignancy in men after non-melanoma skin cancer. Approximately 11.700 men are diagnosed yearly in the Netherlands, from which 2800 men die because of this disease. Current markers for PCa, such as PSA, have a good sensitivity but lack specificity. Also, PSA-based screening leads to a high risk of overdiagnosis and overtreatment. Therefore, new molecular markers are needed.

Chapter 1 presents the general introduction and objectives of this thesis. This chapter gives an explanation on what defines a tumor marker and what types of markers are available. Also, the source of biomarkers (tissue/fluid) is discussed with its advantages and disadvantages. The process with subsequent steps that are needed for a marker to become a clinically useful marker are explained.

When looking for new markers for PCa, it is important to know which markers already have been identified. **Chapter 2** provides an overview of all the markers that were already found when we started our research. The most widely accepted and commonly used marker PSA, but also its isoforms and PSA characteristics are discussed. Other interesting candidate markers are reviewed and extracellular vesicles (exosomes) are introduced.

The search for novel biomarkers has been the focus of many research groups, and the studies have become more extensive and sophisticated. Although exciting progress has been made with respect to novel technologies, discovery of protein markers in bodily fluids such as serum remains difficult. One of these problems is the presence of high abundant proteins which make discovery of promising low abundant proteins challenging (dynamic range problem). Even with current technologies, discovering biomarkers is still like searching for a needle in a haystack. This problem can be partially tackled by profiling of subfractions of bodily fluids such as extracellular vesicles (EVs, also referred to as exosomes). **Chapter 3** gives an overview of relevant data that had been published at the start of the project regarding EVs, including their biogenesis, function and content in relation to PCa. The exact biogenesis of exosomes remains unclear, but they are formed inside a multi-vesicular body (MVB) and released when the MVB merges with the cell membrane. EVs have a wide range of function, mainly in the immune system. By presenting antigens on their membrane they can interact with recipient cells. Furthermore, they have been shown to be able to transport proteins and RNAs. In the field of oncology much debate is going on about the pro- or antitumor effect. Most important techniques for isolation are ultracentrifugation, precipitation and immunoaffinity capture. Reports about profiling PCa-derived EVs are limited, but some have been profiled and this has led to the identification of some candidate markers. Although the number of publications is increasing, further investigations are required to assess the exact clinical values of EVs in prostate cancer.

Profiling content from PCa-derived EVs could reveal novel biomarkers. In **chapter 4** we report on proteomic profiling of PCa EVs. In collaboration with the Environmental

Molecular Science Laboratory (EMSL) in Richland, WA, USA, we analysed EVs from two immortalized primary prostate epithelial cells (PNT2C2 and RWPE-1) and two PCa cell lines (PC346C and VCaP) by using a nanoLC-LTQ-Orbitrap operated in tandem MS (MS/MS) mode. With this approach we identified 52 proteins that were statistically significantly different expressed, from which nine more abundant in EVs from PCa cell lines. From this list, three candidate markers (FASN, XPO1 and PDCD6IP) were selected. Validation by Western blotting confirmed their higher expression in PCa EVs. Interestingly, with immunohistochemistry we noticed that when Gleason score increased, the expression of XPO1 gradually went from the nucleus to the cytoplasm.

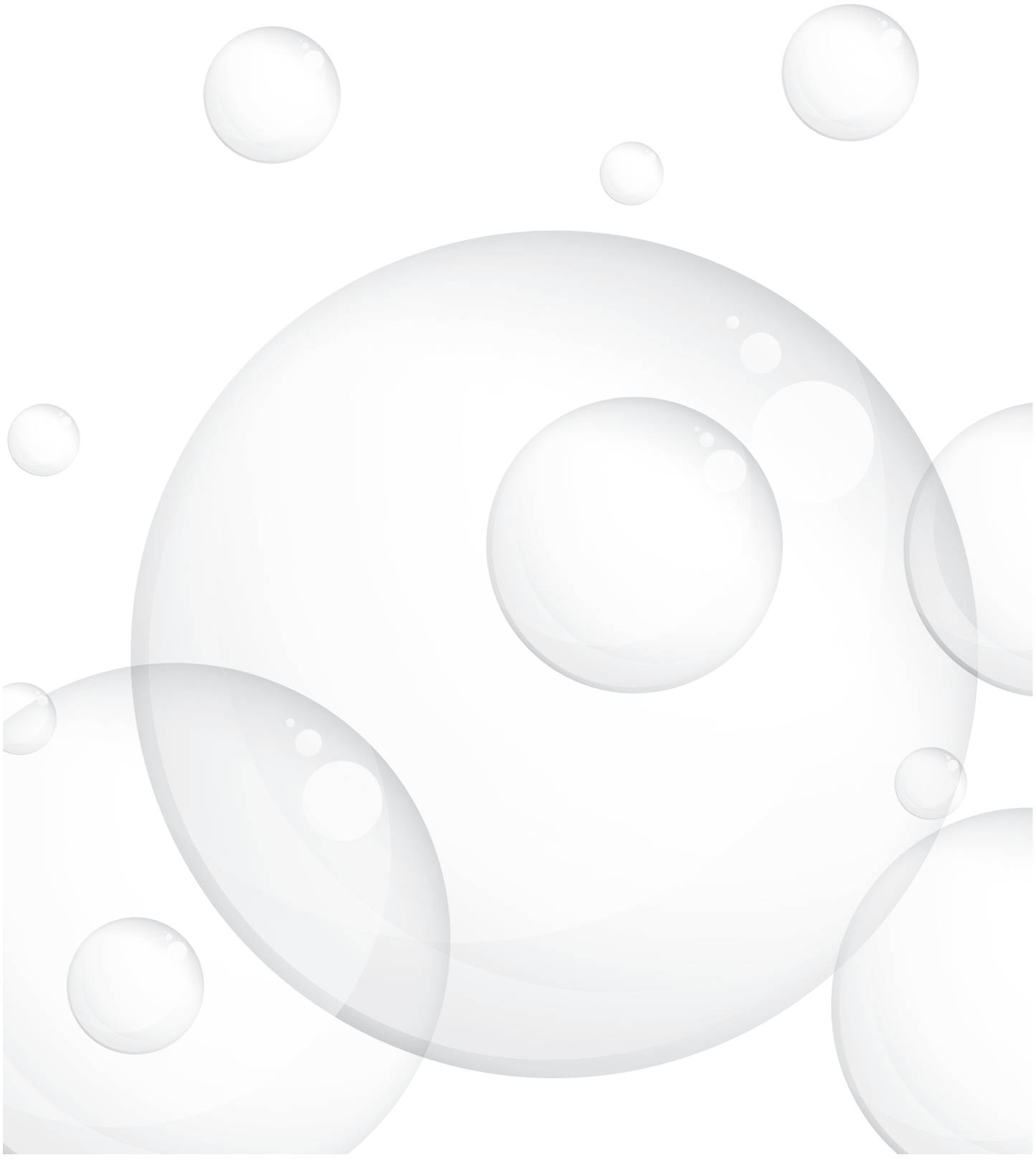
As described in the introduction, in order to elucidate the potential diagnostic and prognostic value of identified candidate markers, they need to be tested on patient samples. **Chapter 5** describes the validation of FASN, XPO1 and PDCD6IP on large well-characterized patient cohorts. First, protein fractions from RNA isolations from 67 samples (33 healthy individuals and 34 PCa patients) were analyzed by nanoLC-MS. Only XPO1 showed higher expression in PCa ($p>0.0001$). Subsequently we used a tissue micro array (TMA) containing 481 patient samples and immunohistochemically stained them for all three markers. When we correlated expression with multiple clinicopathological parameters, only high cytoplasmic XPO1 was correlated with high Gleason score ($p=0.002$) and prostate cancer related death ($p=0.009$). With this study we showed that XPO1 remains an interesting candidate marker for PCa.

In **chapter 6** we used the same clinical samples as in chapter 5 for discovery of protein markers that are able to predict biochemical recurrence (BCR) after radical prostatectomy (RP), with a special interest in proteins that are involved in the arachidonic acid (AA) pathway. After proteomic profiling, 798 proteins were statistically significant expressed between samples from healthy men and men with PCa. From this list four proteins that were dysregulated in PCa, were selected for validation (AGR2, FASN, LX15B and LOX15). Immunohistochemistry showed that AGR2, LOX5 and LX15B was positive in PCa and negative/low positive in normal tissue. FASN showed higher expression when Gleason scores increased. When the same markers were validated on the TMA we observed that AGR2 expression was correlated with Gleason score ($p=0.032$). LOX5 expression in the cytoplasm was associated with higher pT stage ($p=0.044$). FASN showed no clinicopathological correlation. Kaplan-Meier curves revealed that <100% of positive tumor cells for AGR2 (HR (95% CI) = 0.61 (0.43-0.93) and also when LOX5 expression is present (HR (95% CI) = 2.53 (1.23-5.22) are predictors of BCR after RP.

Quantifying the number of EVs and characterizing them on single particle level remains challenging. Most techniques for isolation and characterization are labour intensive and limited with respect to efficient isolation or purity of the final EV preparation. In order to measure tissue-specific EVs from bodily fluids novel assays have to be developed. One technique that is of special interest is an immunoaffinity assay directed

against transmembrane proteins on EVs. In **chapter 7** we describe the development and validation of a highly sensitive TR-FIA (time resolved fluorescence immunoassay) against CD9 and CD63. Cell medium from 37 cell lines and urine from patients with PCa (n=67), men without PCa (n=76) was analysed. As a control, urine was collected from men after radical prostatectomy (n=13), women (n=16) and patients with PCa without digital rectal exam (n=16). After optimisation, we showed that this TR-FIA was able to measure EVs with very high sensitivity and low background signals. CD9 and CD63 are present on EVs from all cell lines with huge variation between them. After correction of urinary PSA as surrogate for the amount of prostate fluid in the urine, expression of CD9 and CD63 was higher in patients with PCa. More PCa-specific antibodies need to be tested using this TR-FIA to discover the most optimal combination of diagnostic and prognostic PCa markers.

In this thesis we have shown that EVs, based on their biogenesis, are an interesting and valuable source for biomarker discovery. Proteomic profiling of PCa EVs led to the identification of three biomarkers. When validated on different independent patient sample cohorts, XPO1 and CD63/PSA remained as the most promising candidate biomarkers. More research is needed in order to fully elucidate it clinical potential. Unfortunately, current techniques for isolation and characterization of EVs are labour intensive, interfere with integrity/purity and lack scalability. Therefore, we developed a highly sensitive TR-FIA that was able to distinguish PCa from healthy men by applying 100 uL urine. More PCa specific antibodies need to be tested to create an assay that has higher sensitivity and specificity. With such an assay, EVs in urine of serum could be used as a diagnostic, prognostic and disease monitoring markers.



Samenvatting

Prostaatkanker (PK) is de op twee na meest voorkomende maligniteit onder mannen. Jaarlijks krijgen 11.700 mannen in Nederland deze diagnose, waarvan ongeveer 2.800 hieraan uiteindelijk overlijden. Huidige markers voor het opsporen van de ziekte, zoals bijvoorbeeld PSA, hebben een goede sensitiviteit, maar hebben te weinig specificiteit. Hierdoor krijgen te veel mannen onnodig de diagnose en bestaat er een kans op overbehandeling. Hierdoor zijn er nieuwe markers nodig die beter in staat zijn om de diagnose te stellen en prognose te bepalen.

Hoofdstuk 1 geeft een algemene introductie weer en bespreekt de doelstelling van dit proefschrift. Verder geeft dit hoofdstuk uitleg over wat een marker precies is en welke types er zijn. Ook de bron van deze marker (urine of bloed) wordt bediscussieerd. Stappen in de ontwikkeling van een marker naar de kliniek worden uiteengezet.

Wanneer men zoekt naar een nieuwe marker voor PK, is het belangrijk om te weten welke er al zijn beschreven in de literatuur. **Hoofdstuk 2** geeft een overzicht van alle markers voor PK die tot dat moment bekend waren. In dit hoofdstuk wordt naast PSA (inclusief varianten en karakteristieken) en andere beschreven markers, blaasjes ofwel 'extracellular vesicles' (EVs) geïntroduceerd.

Wereldwijd zijn er veel onderzoeksgroepen die zich richten op het ontdekken van nieuwe markers voor PK. Ondanks de vooruitgang omtrent technische ontwikkelingen, blijft het ontdekken van deze markers uit bloed of urine een uitdaging. Een van de voornaamste problemen is het zogenaamde 'dynamic range problem'. In urine en bloed zitten veel verschillende eiwitten en RNAs in sterk verschillende concentraties. De meest interessante markers zijn waarschijnlijk in een zeer lage concentratie aanwezig. Hierdoor worden ze overschaduwd door de veel voorkomende eiwitten/RNAs en kan men deze interessante markers lastig identificeren. Het is net als zoeken naar een 'naald in een hooiberg'. Eén van de oplossingen zou het onderzoeken van EVs kunnen zijn. **Hoofdstuk 3** geeft een overzicht van alle publicatie omtrent EVs, inclusief biogenese, functie en inhoud in relatie tot PK. Hoe EVs gemaakt worden is nog niet helemaal duidelijk, maar wel weten we dat ze worden gemaakt in zogenaamde 'multi-vesicular bodies (MVB)'. Nadat deze MVB op gaat in het celmembraan, komen de EVs vrij. Buiten de cel hebben ze allerlei functies, voornamelijk in het afweersysteem. Door het presenteren van antilichamen kunnen ze interactie hebben met de ontvangende cel. Daarnaast kunnen ze eiwitten en RNA transporteren van de ene naar de andere cel. Binnen de oncologie is de rol van EVs nog onduidelijk waarbij ze soms pro- of antitumor effecten hebben. De belangrijkste technieken voor isolatie zijn ultracentrifugatie, precipitatie en via immuunaffiniteit. Het aantal publicaties over EVs en PK zijn beperkt, echter sommige hebben al kandidaat markers gevonden voor deze ziekte. Ook al neemt het aantal publicaties over dit specifieke onderwerp toe, er is meer onderzoek nodig om de klinische waarde van EVs beter te bepalen.

Omdat EVs door hun biogenese een weergave zijn van de cel waarvan ze afkomstig zijn, zou het in kaart brengen van hun inhoud kunnen leiden tot het vinden van nieuwe markers. In **hoofdstuk 4** hebben we eiwitten in EVs onderzocht. In samenwerking met het Environmental Molecular Science Laboratory (EMSL) in Richland, WA, USA, hebben we EVs geanalyseerd van twee prostaat epitheel cellijnen (PNT2C2 en RWPE-1) en twee PK-cellijnen (PC346C en VCaP) door een nanoLC-LC-LTQ-Orbitrap massaspectrometer te gebruiken. Met deze techniek hebben we 52 eiwitten gevonden die verschillend tot expressie kwamen, waarvan er negen hoger tot expressie kwamen in PK EVs. Van deze lijst werden drie kandidaat markers geselecteerd (FASN, XPO1 en PDCD6IP). Validatie met behulp van Western blotting bevestigde de hogere expressie in EVs afkomstig van PK. Wanneer we deze markers valideerden middels immuunhistochemie viel het op dat wanneer de Gleason score omhoogging, de expressie van XPO1 zich van de kern verplaatste naar het cytoplasma. Dit gaf het idee dat translocatie van XPO1 geassocieerd zou kunnen zijn aan een slechtere klinische uitkomst.

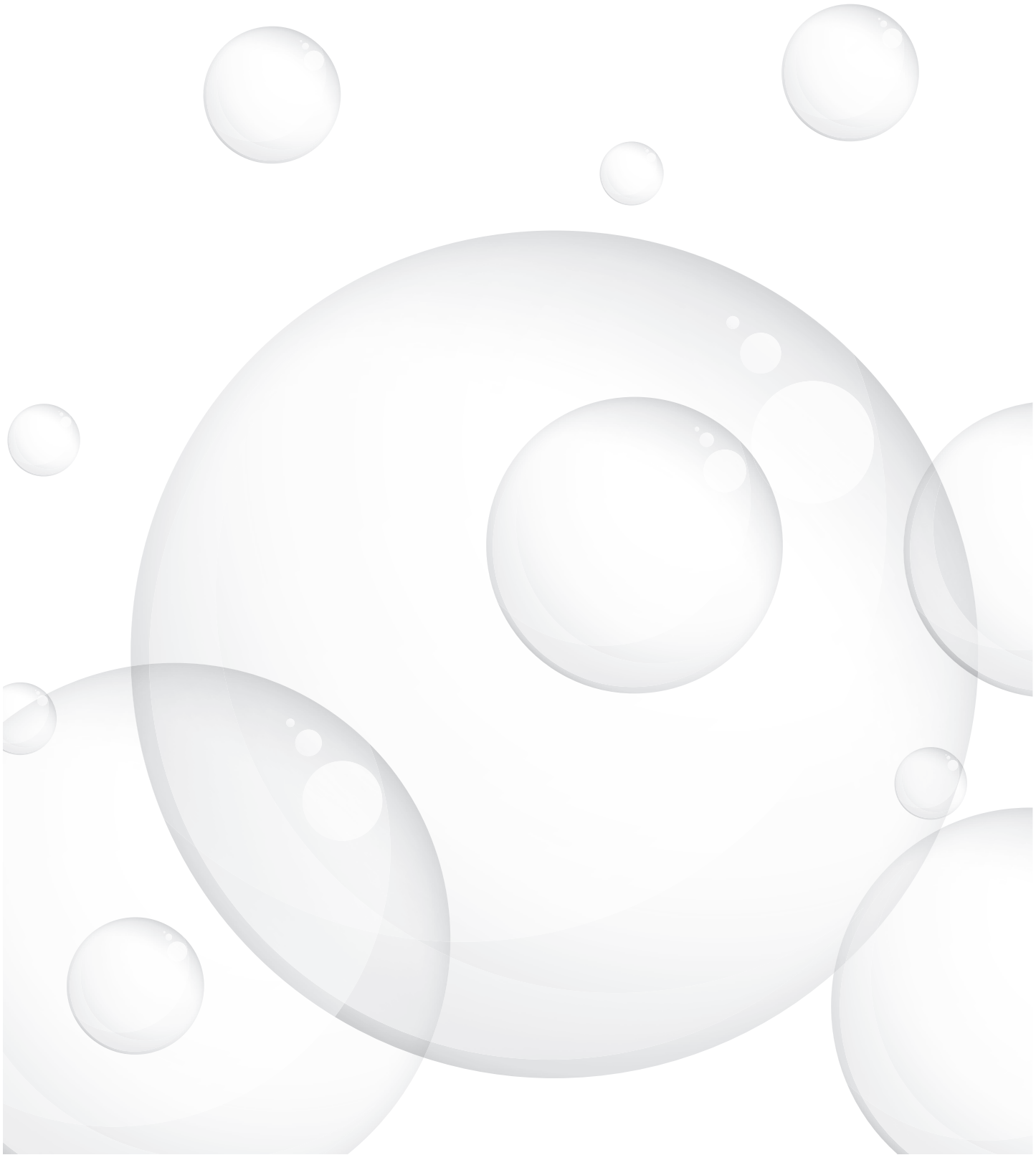
Zoals beschreven in de introductie, moet men om de vertaling te maken van ontdekking naar een klinisch toepasbare marker, deze getest worden op patiëntmateriaal. **Hoofdstuk 5** beschrijft de studie waarin we de drie eerder ontdekte kandidaat markers (FASN, XPO1 en PDCD6IP) hebben gevalideerd op materiaal van twee onafhankelijke grote groepen patiënten. In eerste instantie hebben we eiwitfracties van weefsel (na RNA-isolatie) genomen van 67 patiënten (33 gezonde mannen en 34 PK-patiënten) en deze geanalyseerd met een nano-LC massaspectrometer. In deze studie liet alleen XPO1 een hogere expressie zien in PK ($p>0.0001$). Hierna hebben we dezelfde markers immunohistochemisch getest in een 'tissue microarray' (TMA) die weefsel bevatte van 481 patiënten na radicale prostatectomie. Wanneer we de expressie vergeleken met meerdere klinische en pathologische uitkomsten was alleen XPO1 in cytoplasma gecorreleerd aan een hoge Gleason score ($p=0.002$) en aan PK-gerelateerd overlijden ($p=0.009$). Met deze studie hebben we laten zien dat XPO1 een interessante marker is voor PK.

In **hoofdstuk 6** hebben we dezelfde klinische validatie sets gebruikt als in hoofdstuk 5 om zo eiwitten, betrokken binnen het 'Arachidonic acid pathway', te valideren ten aanzien van een biochemisch recidief na radicale prostatectomie. In totaal werden 798 eiwitten gevonden die statisch significant verschilden tussen gezond weefsel en PK. Uit deze list werden vier eiwitten gekozen die verhoogd tot expressie kwamen in PK (AGR2, FASN, LX15B en LOX15). Immuunhistochemie toonde dat AGR2, LOX5 en LX15B positief waren in PK en negatief/laag positief in normaal weefsel. FASN kwam hoger tot expressie wanneer de Gleason score toename. Wanneer deze markers werden getest op de TMA, was AGR2 gecorreleerd aan een hogere Gleason score ($p=0.032$). LOX5 expressie in het cytoplasma bleek geassocieerd aan een hogere klinisch pT-stadium ($p=0.044$). FASN liet geen relatie zien met klinische of pathologische parameters. Kaplan-Meier analyse

toonde dat een laag percentage met positieve tumorcellen (<100%) voor AGR2 (HR (95% CI) = 0.61 (0.43-0.93) en ook aanwezige LOX5 expressie (HR (95% CI) = 2.53 (1.23-5.22) voorspellers waren voor een biochemisch recidief na radicale prostatectomie.

Kwantificatie van EVs en het karakteriseren op het niveau van een enkel blaasje blijft zelfs met nieuwe ontwikkelingen, zeer uitdagend. De meeste technieken die nu worden toegepast zijn tijdsintensief en beperkt in hun efficiëntie/behoudt van integriteit van de EVs. Om weefselspecifieke EVs uit urine of bloed te meten, moeten nieuwe en betere assays ontwikkeld worden. Een interessante techniek waarmee isolatie en karakterisatie in één assay gecombineerd zou kunnen worden is een (sandwich)-immuunaffiniteits assay gericht tegen transmembraameiwitten die tot expressie komen op EVs. In **hoofdstuk 7** beschrijven we de ontwikkeling en validatie van een hoog-sensitieve TR-FIA ('time resolved fluorescence immunoassay') tegen de transmembraameiwitten CD9 en CD63. Kweekmedium van 37 verschillende cellijnen en urine van PK-patiënten (n=67), mannen zonder PK (n=76). Ter controle namen we urine van mannen na een radicale prostatectomie (n=13), vrouwen (n=16) en patiënten met PK, maar dan zonder prostaatmassage (n=16). Na optimalisatie toonde we aan dat deze assay met een hoge sensitiviteit en een zeer laag achtergrondsignaal, EVs kon meten. De expressie van CD9 en CD63 varieerde enorm tussen de verschillende cellijnen. In de klinische samples was de expressie van CD9 en CD63 hoger in urine van PK-patiënten (na correctie voor PSA in de urine). Meer PK-specifieke moeten in deze assay getest worden om zo de meest ideale combinatie voor diagnose en prognose voor PK te bepalen.

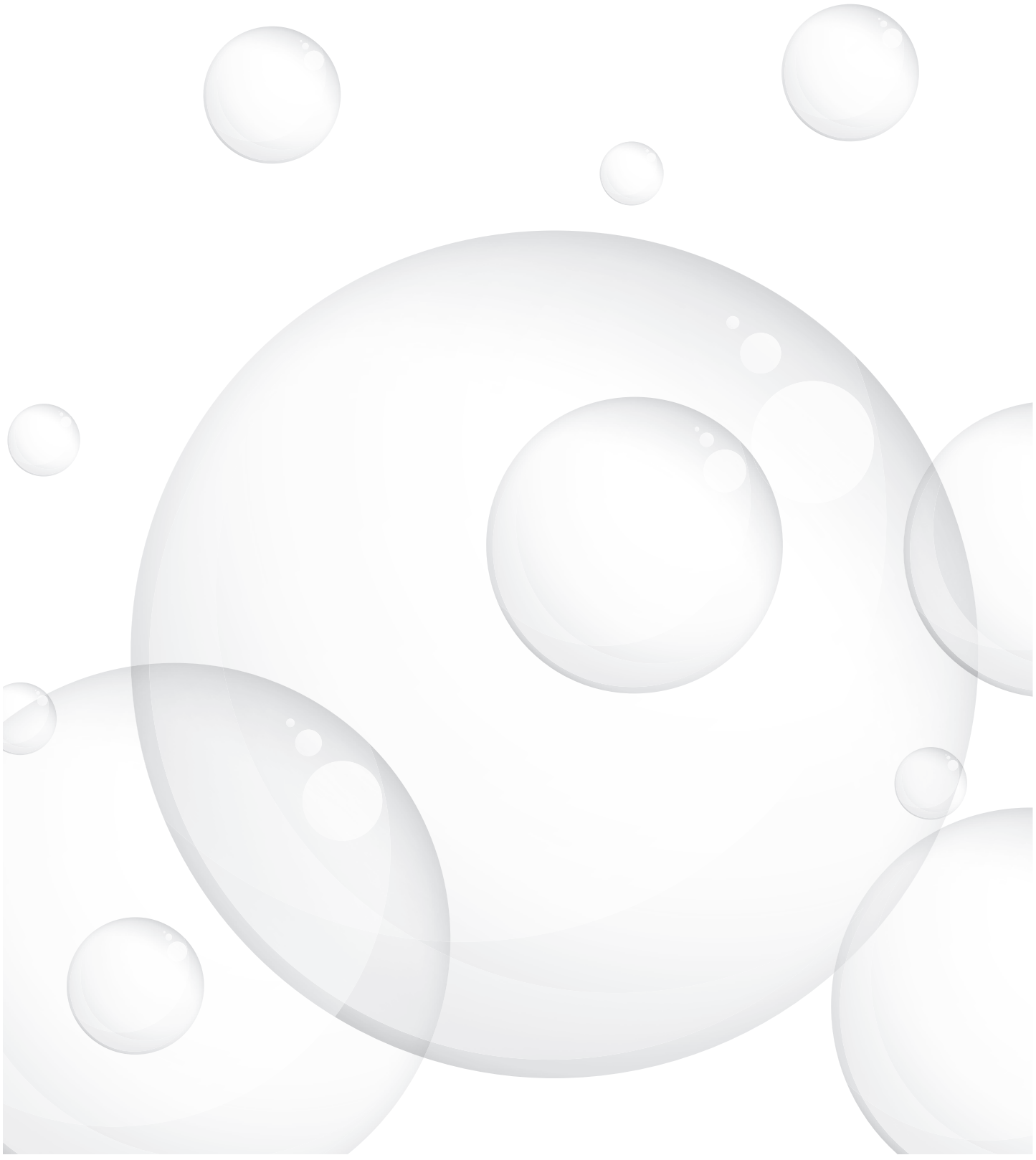
In dit proefschrift hebben we laten zien dat EVs, vanwege hun biogenese, een interessante bron zijn voor het zoeken naar nieuwe markers voor PK. Het in kaart brengen van PK EVs heeft geleid tot de identificatie van meerdere kandidaat markers, waarvan XPO1 en CD63/PSA overbleven na validatie op klinische patiënt weefsel. Meer onderzoek is nodig om de exacte klinische toepasbaarheid van deze marker vast te stellen. Omdat huidige technieken voor het isoleren en karakteriseren van EVs tijdsintensief zijn en niet in staat zijn om grote hoeveelheden samples te verwerken, moeten nieuwe technieken ontwikkeld worden. Wij hebben succesvol een zeer sensitieve TR-FIA ontwikkeld die in staat was om EVs te meten en onderscheid te maken tussen PK en gezonde mannen. Meer PK-specifieke antilichamen moeten in deze assay getest worden om een nog betrouwbaardere diagnose te stellen en de prognose beter te voorspellen. Met deze assay zouden EVs in de toekomst gebruikt kunnen worden als marker voor diagnostiek, prognose en ziekte beloop.



Abbreviations

AA	Arachidonic acid
AGR2	Anterior gradient 2
AMACR	Alpha-methylacyl coenzyme A racemase
AMT	Accurate mass and time
APC	Antigen presenting cell
AUC	Area under curve
BCR	Biochemical recurrence
BPH	Benign prostate hyperplasia
BSA	Bovine serum albumin
CAV1	Caveolin-1
CM	Confocal microscopy
CRISP3	Cysteine-rich secretory protein 3
CRM1	Chromosomal maintenance 1
CRPC	Castration resistant prostate cancer
DNA	Deoxyribonucleic acid
DRE	Digital rectal exam
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
EMSL	Environmental and molecular science laboratory
ENO1	Enolase-1
ERSPC	European randomized study of screening of prostate cancer
ESCRT	Endosomal sorting complex responsible for transport
Eu	Europium
EV	Extracellular vesicles
FACS	Fluorescence-activated cell sorting
FASN	Fatty acid synthetase N
FCS	Fetal calf serum
FDR	False discovery rate
FFPE	Formalin-fixed paraffin-embedded
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GOLPH2	Golgi phoshoprotein 2
GSTP1	Glutathione S-transferase pi 1
GWAS	Genome wide association studies
HETE	Hydroxyeicosatetraenoic acid
HPRD	Human protein reference database
HRP	Horse radish peroxidase
IHC	Immunohistochemistry
IPA	Ingenuity pathway analysis

KLK2	Kallikrein 2
KLK3	Kallikrein 3
LC	Liquid chromatography
LDHA	Lactate dehydrogenase-A protein
LFQ	Label free quantification
LOX5	Lipoxygenase 5
LX15B	Arachidonate 15-lipoxygenase type B
MHC	Major histocompatibility complex
mRNA	Messenger RNA
MS	Mass spectrometry
MVB	Multivesicular body
MYO6	Myosin 6
NAP	Normal adjacent prostate
P/S	Penicillin/Streptomycin
PBS	Phosphate buffered saline
PSA	Prostate specific antigen
PCa	Prostate cancer
PCA3	Prostate cancer antigen 3
PDCD6IP	Programmed cell death 6 interacting protein
PRM	Parallel reaction monitoring
PSCA	Prostate stem cell antigen
PSMA	Prostate specific membrane antigen
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
RP	Radical prostatectomy
RPLC	Reverse phase liquid chromatography
RT-PCR	Reverse transcriptase PCR
SNP	Single nucleotide polymorphisms
TMA	Tissue micro-array
TRF	Time resolved fluorescence
TR-FIA	Time resolved fluorescence immunoassay
TURP	Transurethral resection of the prostate
UCr	Urinary creatinine
UPSA	Urinary prostate specific antigen
XPO1	Exportin-1



List of publications

Duijvesz D, Luider TM, Bangma CH, Jenster G. *Exosomes as biomarker treasure chests*. Eur Urol. 2011. 59(5):823-31

Duijvesz D**,** Jenster G, Tumor markers. In: Tewari A. (eds) Prostate Cancer: A comprehensive perspective. Springer, London. 2013. 423-444.

Duijvesz D**,** Burnum-Johnson KE, Gritsenko MA, Hoogland AM, Vredenbregt-van den Berg MS, Willemsen R, Luider TM, Pasa-Tolic L, Jenster G. *Proteomic profiling of exosomes leads to the identification of novel biomarkers for prostate cancer*. PLoS One. 2013. 8(12):e82589

Duijvesz D*, Versluis CY*, van der Fels CA, Vredenbregt-van den Berg MS, Leivo J, Peltola MT, Bangma CH, Pettersson KSI, Jenster G. *Immuno-based detection of extracellular vesicles in urine as diagnostic marker for prostate cancer*. Int J Cancer. 2015. 137(12):2869-78
**authors equally contributed*

Duijvesz D, Rodriguez-Blanco G, Hoogland AM, Verhoef EI, Dekker LJM, van Leenders GJLH, Luider TM, Jenster G. *Differential tissue expression of extracellular vesicle-derived proteins in prostate cancer.* Submitted

Rodriguez-Blanco G*, Zeneyedpour L*, Duijvesz D*, Hoogland AM, Verhoef EI, Kweldam CF, Burgers PC, Sillevis Smitt P, Bangma CH, Dekker LJM, Jenster, G, van Leenders GJLH, Luider TM. *Tissue proteomics outlines AGR2, FASN and members of the arachidonic acid pathway as markers for prostate cancer.* Oncotarget. 2018. 23;9(92):36444-36456
**authors equally contributed*

Publications outside thesis

Lock MTWT, Duijvesz D, van Roijen JH. *Azoospermia: an incorrect prognosis*. NTvU. 2008. 16(6):148-54

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Duijvesz D, Hoekstra RJ, Bosch JLHR. Voiding complaints in a rectus sheeth hematoma. NTvG. 2009. 153:B189

Zhu Y, Duijvesz D, Lock MTWT. *Re: Trevor D. Schuler, Rohan Shahani, R. John D'A. Honey and Kenneth T. Pace. Medical Expulsive Therapy as an Adjunct to Improve Shockwave Lithotripsy Outcomes: A Systematic Review and Meta-Analysis*. J Endourol. 2009. 23(8):1365-6

Steijns JJM, Duijvesz D, Breedijk MA, GMCJ vd Heijden. *Steroid injection in addition to macular laser grid photocoagulation in diabetic macular oedema: systematic review*. Acta Ophthalmol. 2010. 88(4):389-93

Zhu Y, Duijvesz D, Rovers M, Lock MTWT. *Alpha-blockers to assist stone clearance after extracorporeal shock wave lithotripsy: a meta-analysis*. BJUI. 2010. 106(2):256-61

Zhu Y, Rovers MM, Duijvesz D, Lock MTWT. *Alpha-blockers as medical expulsive therapy for ureteral stones*. Cochrane Database of Systematic Reviews 2010, Issue 5. Art. No.: CD008509. Updated in 2014.

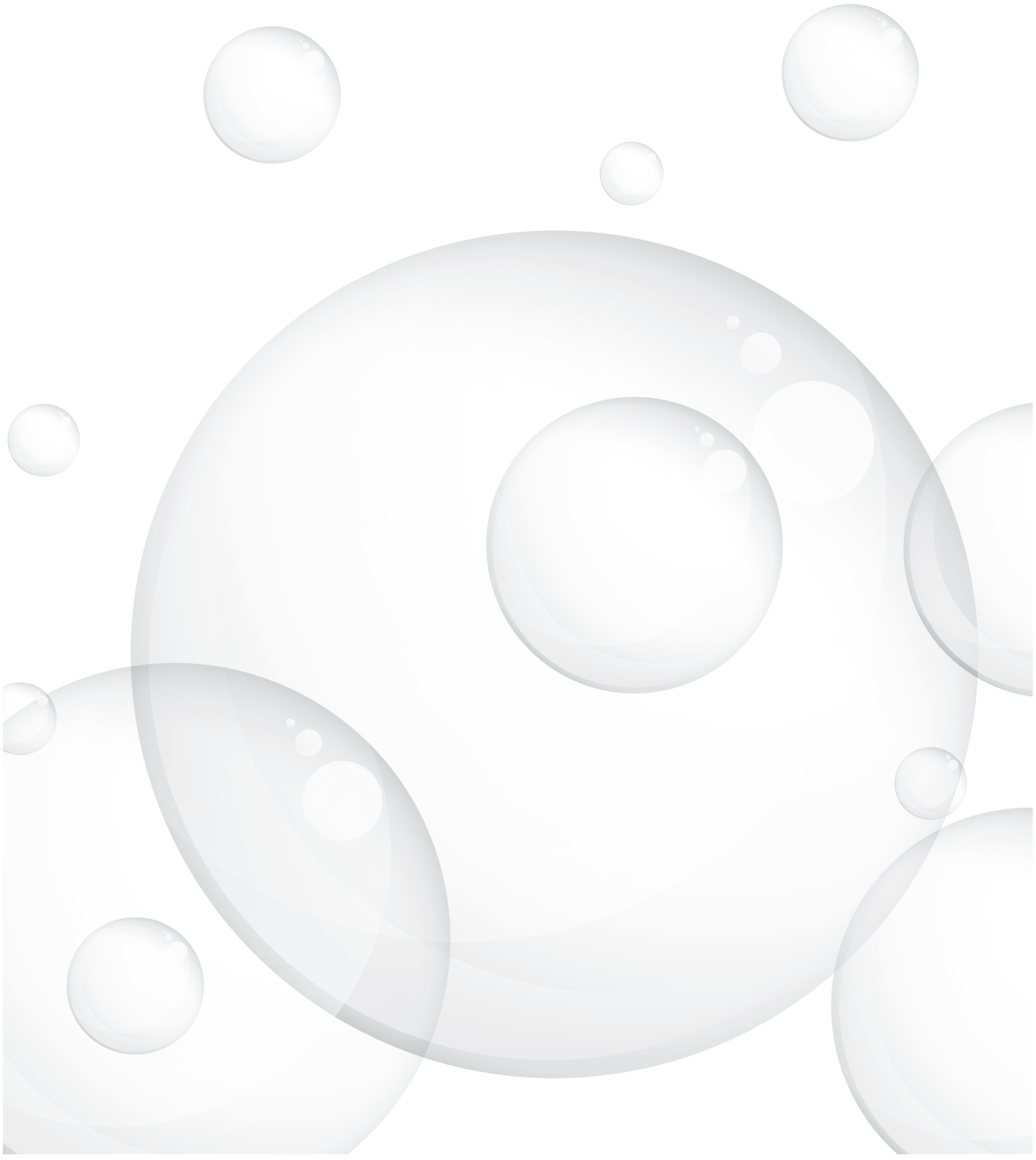
Duijvesz D, Zhu Y, Rovers M, Lock MTWT. *Alpha-blockers as medical expulsive therapy for distal ureteral stones: a meta-analysis*. NTvU. 2010. 18(7):193-200

Duijvesz D**,** Lock MTWT, Kraak R, Verhagen PCM, Bosch JLHR. The *psoas hitch procedure is a reliable method for distal ureteral pathology*. NTvU. 2011. 19(1):2-6.

Lock MTWT, Duijvesz D, Fitzpatrick JM. *Minireview: Ehlers-Danlos: Nobody is perfect*. Chapter book: Ehlers Danlos, to be published.

Zhu Y, Duijvesz D, Rovers M, Lock MTWT. *Evidence-based urology in practice: Publication Bias*. BJUI. 2011. 107(2):337.

Sterenborg TBRM, Tjiam IM, Vreuls W, Duijvesz D. *Case report. Een benigne zwelling van de testis: een intratesticulaire epidermoïdcyste. Cave: overige echografische afwijkingen*. TvU. 2019. 9:16.



Dankwoord

Na ruim 10 jaar kan eindelijk de laatste bladzijde van dit proefschrift geschreven worden. Het is af. Dit werk heeft zich niet vanzelf geschreven waarbij ik tijdens de jaren in het lab, maar ook daarna, veel steun heb gehad. Hierdoor ben ik velen dank verschuldigd. Zonder jullie geen proefschrift!

Geachte prof. dr. ir. Jenster, beste Guido, toen ik voor het eerst bij je op bezoek kwam had ik helemaal niet de wens om basaal/celbiologisch onderzoek te willen gaan doen. Echter vanaf het eerste gesprek werd ik gepakt door jouw enthousiasme. De daaropvolgende jaren van intensieve samenwerking waren voor mij een geweldige ervaring waarin ik alle ruimte kreeg om mezelf te ontdekken en te ontwikkelen op wetenschappelijk niveau. Altijd was er de mogelijkheid tot het stellen van vragen over ons onderzoek, maar je was er ook als ik op zoek was naar een sociaal praatje of motivatie. Dank je wel dat je de gok hebt genomen om mij te willen begeleiden!

Geachte prof. dr. Bangma, beste Chris, tijdens mijn tijd in het Erasmus MC heb ik veel van je mogen leren. Nog steeds verwondert het me dat je een haarfijn gevoel hebt voor kliniek, (basaal) wetenschap en de translatie tussen die twee werelden. Jouw openheid, toegankelijkheid en kritische blik hebben absoluut bijgedragen aan een betere kwaliteit van dit werk. De persoonlijke noot tijdens congressen of op reis ervaarde ik als zeer prettig. Dank je wel voor al je tijd!

Geachte dr. Luider, beste Theo, vanaf het begin van mijn tijd in het lab hebben we regelmatig contact gehad. Mijn kennis over massaspectrometrie was vrijwel gelijk aan nul, maar jij hebt me samen met jouw team hierin begeleidt. Dank je wel voor jouw opmerkingen en stimulerende vragen tijdens het gehele proces.

Beste leden van de leescommissie en promotiecommissie, dank voor jullie kritische beoordeling van mijn proefschrift en jullie bereidheid plaats te nemen als opponent.

Beste co-auteurs, dank voor jullie samenwerking en input. Met z'n allen hebben we toch een fraai stukje werk neergezet.

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Beste collega's van het Erasmus MC en Amphia ziekenhuis, tijdens mijn opleiding ben ik (helaas) niet veel aan wetenschap toegekomen. Daarentegen hebben jullie mij altijd positief gestimuleerd om hier wel mee door te gaan en het toch een keer af te maken. Jullie hebben mijn opleiding leuk en leerzaam gemaakt.

Beste collega's van het CWZ, ook al hebben jullie me weinig meegemaakt tijdens mijn promotie, toch mijn dank voor de fijne start in Nijmegen. Mijn aandachtsgebied is niet meer gerelateerd aan het onderwerp van mijn proefschrift, desalniettemin kijk ik er bijzonder veel naar uit om de komende tijd met jullie te mogen samenwerken.

Geachte drs Lock, beste Tycho, jij stond aan de basis van mijn carrière binnen de urologie. Vanaf dag één heb je mij op veel manieren kennis laten maken met de kliniek, maar ook met de beginselen van het onderzoek doen. Dank je wel voor alle tijd die je voor mij hebt vrijgemaakt.

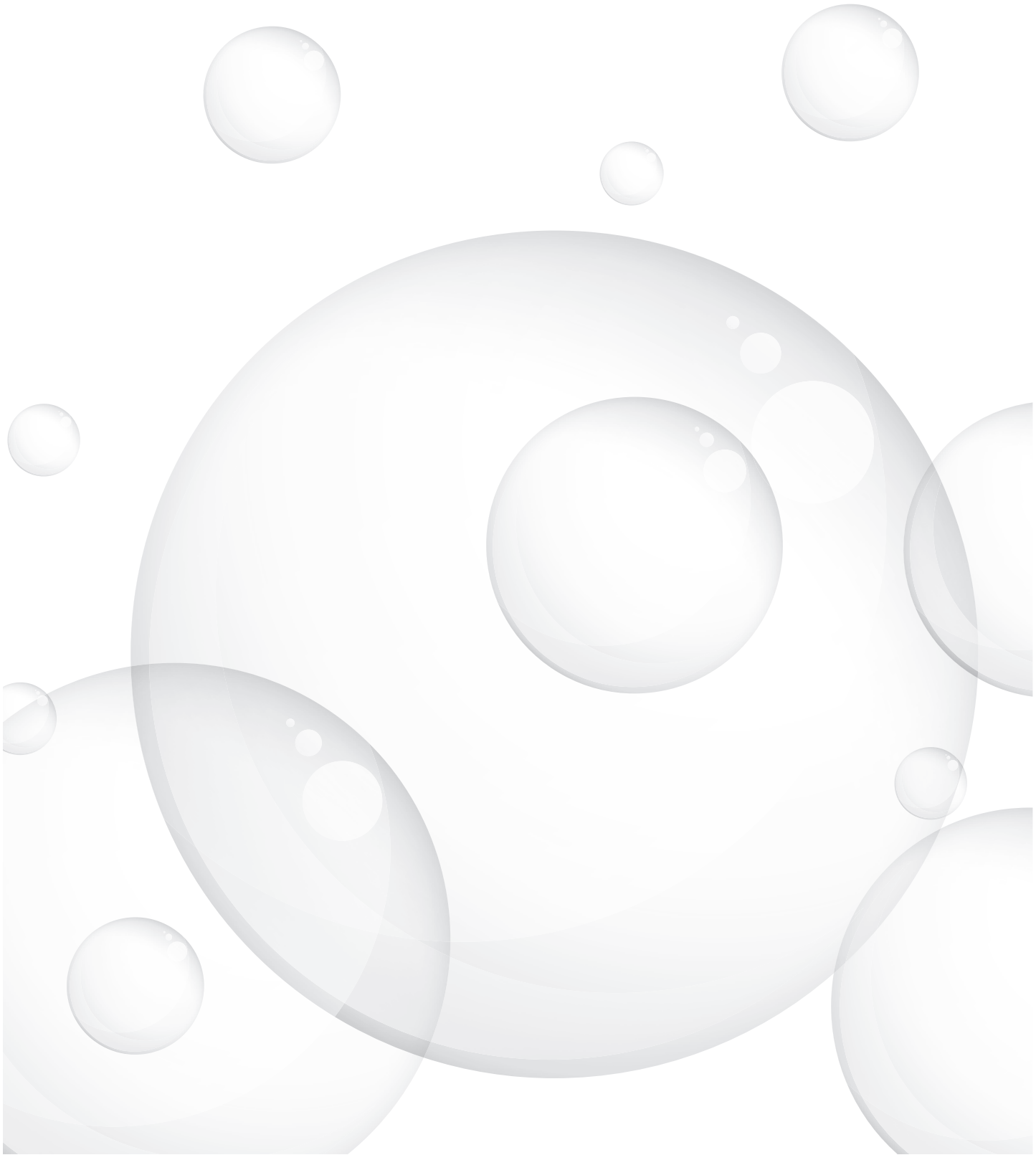
Beste Robert, Dennis en Daan, sinds het begin van Geneeskunde trekken we met elkaar op en zijn we gelukkig nog steeds goed bevriend. We zien elkaar niet zeer frequent, maar de avondjes bier drinken doen mij veel goed. Dank voor jullie vriendschap!

Beste Martijn en Richard, vrienden van het eerste uur. Martijn, jouw voorstel aan mijn ouders om het huis te kopen naast jouw ouders, is een gouden zet geweest. Tijdens al die jaren is er een vriendschap ontstaan die ik bijzonder veel koester. Richard, het begon op de middelbare school, maar onze vriendschap hebben we voortgezet toen we samen in het studentenhuis op de Hessenweg in de Bilt gingen wonen. Vele avonden volgden met eten, bier en eindeloze (onzinnige) discussies. Nog steeds kan ik hier bijzonder veel van genieten. Ik kan me geen fijnere vrienden voorstellen. Voor mij volkomen logisch dat jullie mijn paranimfen zijn!

Lieve Hanneke, Pauline, Jurriaan, Sara, Elise, Alejandro, Lianne, Jantine, Nick en Erik, jullie zijn zondermeer de fijnste schoonfamilie die ik had kunnen wensen. Zo druk als het is als we bij elkaar zijn, zo veel ontspanning geven jullie mij. Gelukkig geen lange gesprekken over mijn onderzoek, maar vooral veel plezier en lekker samen zijn.

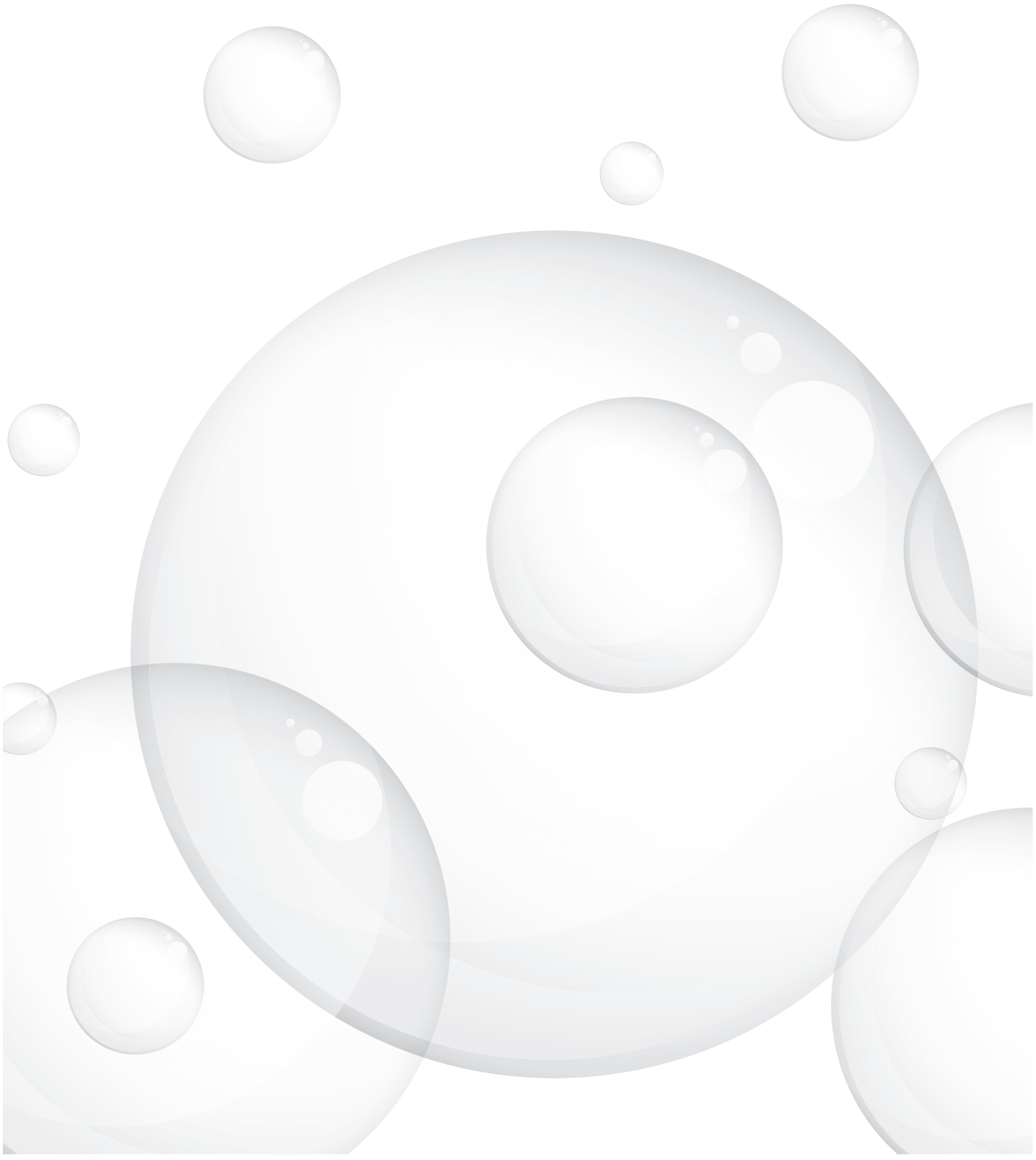
Lieve Mama, Ruud, Yasmijn, Bente, Ramon en Erin, jullie zijn en blijven de basis waar alles begonnen is. Dank voor jullie support. Het is niet altijd even leuk of gemakkelijk geweest, maar met elkaar kunnen we alles aan! Het is een bijzonder prettig gevoel dat ik onderdeel ben van ons gezin.

Lieve Christine, zo onzeker als de toekomst is over de bevindingen in dit proefschrift, zo zeker ben ik over onze toekomst samen. Jij bent en blijft de beste 'ontdekking' die ik ooit heb gedaan. Als mooi resultaat van onze liefde hebben Benjamin, Puk en Dex zich aangesloten bij ons gezin. Jij geeft me de kracht, energie en ruimte om te zijn wie ik ben. Ik ben heel dankbaar dat ik samen met jou mag zijn. Ik hou van je!



Curriculum vitae

Diederick was born on July 19th 1984 in Vlissingen, the Netherlands. He obtained his high school diploma (VWO) in 2002 and started with his medical training at Utrecht University. During medical school he became interested in Urology which resulted in multiple (inter)national publications and a clinical elective Urology at Groote Schuur Hospital, Cape Town, South-Africa. After graduating in December 2008 he started his 3-year PhD training in January 2009 at the Erasmus Medical Center under supervision of prof. dr. ir. Jenster. During his PhD training he worked in the US and Finland and has given multiple national and international presentations. At the end of his PhD training he worked as a medical doctor at the department of Urology at the Erasmus Medical Center. Subsequently he was accepted for his Urology training. From 2012-2013 he worked at the department of general surgery at the Sint Franciscus Gasthuis in Rotterdam (supervisor: dr. G.H. Mannaerts). The academical part of his Urology training was performed from 2014-2015 at the department of Urology at the Erasmus Medical Center in Rotterdam (supervisor: dr. P.C.M.S Verhagen). From 2016-2017 he completed his training at the department of Urology at Amphia Hospital in Breda (supervisor: dr. D.K. van der Schoot). Throughout his Urology training he has also worked as treasurer and secretary of the European Society of Residents in Urology (ESRU). From July 1st 2017 he began his career as a Urologist at the Canisius Wilhelmina Hospital in Nijmegen, where he focusses on endo-urology, stone treatment and laparoscopy.



PhD portfolio

Name PhD student: Diederick Duijvesz	**PhD period:**	January 2009 – December 2011
Erasmus MC Department: Urology	**Promoter:**	prof. dr. ir Jenster
Research Schools: Molecular Medicine (MolMed)	**Co-promotor:**	dr. Luider

	Year	Workload (ECTS)
General courses		
Biomedical research techniques	2009	1.6
Course Advanced Medical Imaging (AMIE) for physicians	2009	0.25
Course Scientific English Writing	2010	4.0
Workshop Photoshop CS3	2010	0.25
Course Research Management	2010	1.0
Course Integrity in Research	2010	2.0
Workshop Successful Grant Writing	2010	0.5
Course Introduction to Data-analysis (NIHES)	2010	1.0
Course Basic and Translational Oncology	2010	1.8
Training outside department		
3-week training mass spectrometry, EMSL, Richland, Washington VS	2009	3.0
2-week training immune affinity assays, Department Diagnostics Technologies and Applications, Turku, Finland	2011	2.0
Seminars and workshops		
JNI oncology lectures	2009-2011	2.0
Department Journal Club	2009-2011	2.0
Department research presentation / PhD meeting	2009-2011	2.0
Department refereeravond	2009-2011	2.0
Symposium 'Multidisciplinaire behandeling prostaatkanker'	2009-2011	0.25
Urology EAU and AUA review meetings	2009-2011	1.0
NVU voor- en najaarsvergadering	2009-2011	1.0
Presentations		
Oral presentation at Josephine Nefkens Institute (JNI), three times	2010-2011	1.0
Oral presentation scientific lab meetings, 12 times	2009-2011	5.0
Oral presentation lecture evening Erasmus MC (for medical doctors), two times	2010-2011	1.0
Oral presentation PhD meeting urology (promovendi-avond), two times	2010-2011	1.0
Oral presentation 'Wetenschapslunch' Urology & Gynaecology	2010	0.5
Invited lecturer for course 'Biomarker Discovery using old and new technologies', Turku, Finland	2010	0.5
International conferences		

Poster presentation, PCTRE, Amsterdam, the Netherlands	2009	0.5
Poster presentation, SBUR, New Orleans, USA	2009	1.0
Poster presentation, MolMed-day, Rotterdam, the Netherlands	2010	0.5
Poster presentation, ESUR, Vilnius, Lithuania	2010	0.5
Micro and nanovesicles, Oxford, UK	2010	0.5
Poster presentation, KWF SOTO, Ede, the Netherland	2010	0.5
Oral presentation, SEOHS, Rotterdam, the Netherland	2010	0.5
Oral Presentation, IWE, Paris, France	2011	0.5
Oral and poster presentation, EAU, Vienna, Austria	2011	1.0
Poster presentation, WBUR, Innsbruck, Austria	2011	1.0
Oral presentation, EAU, Paris, France	2012	1.0
Poster presentation, AUA, Atlanta, USA	2012	1.0
Oral and poster presentation, ISEV, Gothenborg, Sweden	2012	1.0
Poster presentation, ISEV, Rotterdam, the Netherlands	2014	0.5
Oral and poster presentation, EAU, Madrid, Spain	2016	1.0

Teaching activities

Yin Versluis, student master Molecular Science, 1 year	2010	5.0
Nilab Naïma, HLO-student, 7 months	2011	3.0
Saeid Alinezhad, PhD-student, 2 weeks	2011	0.5
Liton Ferdhon, PhD-student, 2 weeks	2011	0.5
Course 'anatomy of the male urological and reproductive tract' for medical students	2010-2011	1.5
Course 'basic research' for medical students	2010	0.5
Teaching urological procedures to OR staff	2009-2015	2.0

Extra activities

MolMed day, organizing committee	2010-2011	3.0
PhD committee MolMed school	2010-2011	1.0
Educational committee MolMed school	2010-2011	1.0
Board European Society of Residents in urology (ESRU) Treasurer (2013-2016) and secretary (2017-2018)	2013-2018	
JNI party committee	2010	
Organization departmental 'day-out'	2011	
Participation Alpe d'HuZes fundraising activity, Alpe d'Huez, France	2011	

Awards

KWF-Travel grant	2009
EMSL 1 year extension award	2009
Rene Vogels Stipendium Travel grant	2010

EMSL 1 year extension award	2010
Nominated for PhD of the year	2010
Publication of the year EAU – Residents corner (Duijvesz D. et al, Eur Urol. 2011. 59(5):823-31)	2012