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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