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.