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General introduction and outline thesis

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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.

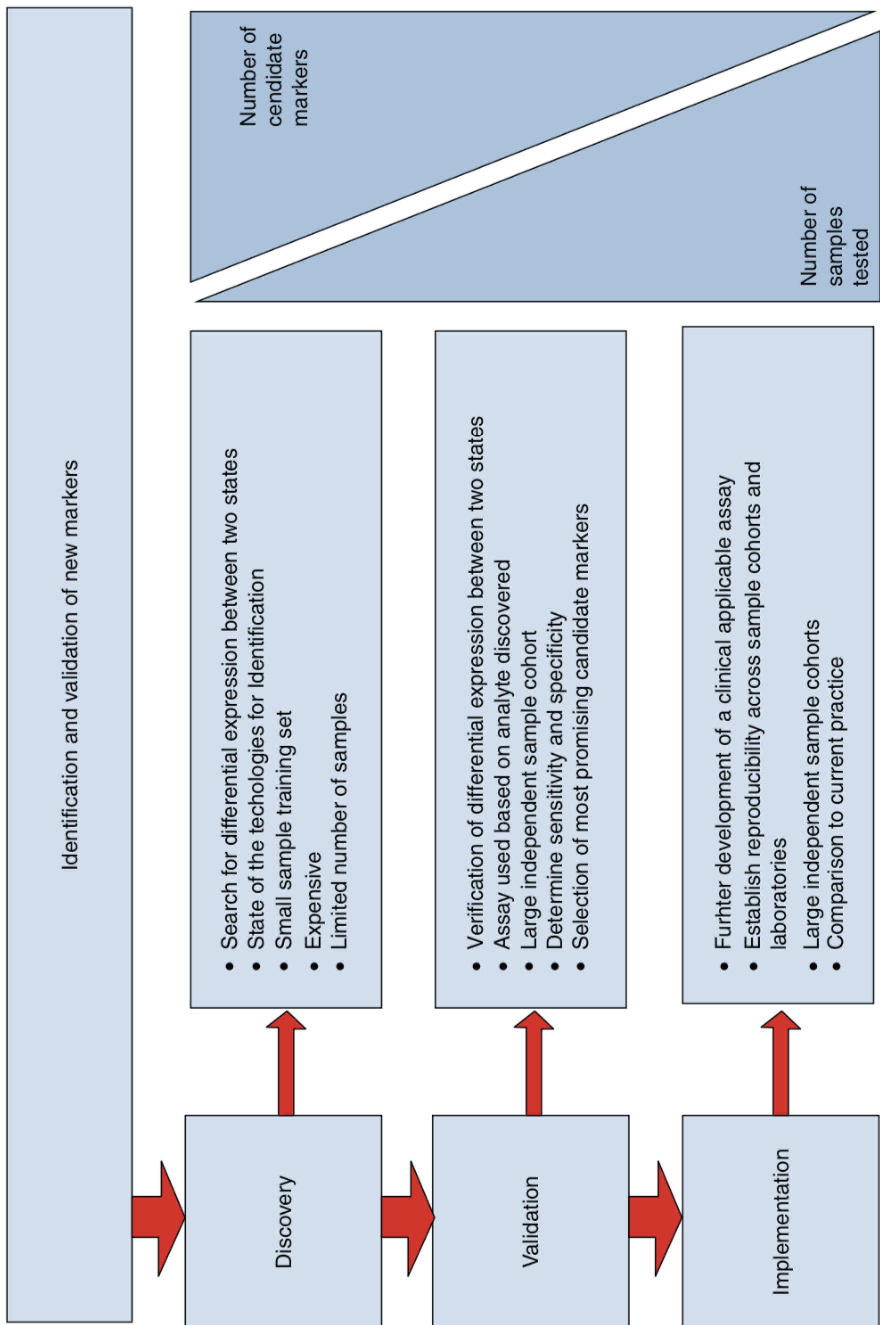

Figure 1. Identification and validation of new markers

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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