Discovery and Validation of prostate cancer biomarkers

DISCOVERY AND VALIDATION OF PROSTATE CANCER BIOMARKERS

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CHAPTER 1

GENERAL INTRODUCTION AND SCOPE OF THE THESIS

Partly derived from: Screening for prostate cancer in 2008 II: the importance of molecular subforms of prostate-specific antigen and tissue kallikreins. European Urology. 2009 March;55(3):563-74*

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1. The prostate

The prostate, derived from the Greek word προστάτης – prostates, meaning "the one who stands before", is a walnut-sized exocrine gland, part of the male genitourinary tract. It produces and stores an alkaline fluid, which liquefies the semen and prolongs the life-span of the spermatozoa. Anatomically, the prostate is located underneath the bladder and in front of the rectum, surrounding the urethra. The prostate can be divided into four distinct zones: the peripheral zone, the central zone, the transition zone and the anterior fibromuscular zone. The majority of prostate cancers (PCa) originate from the peripheral zone. The transition zone is responsible for the disease of benign prostatic hyperplasia (BPH)[1].

2. Prostate-specific antigen

The epithelial cells of the prostate gland produce prostate-specific antigen (PSA), a 34kD glycoprotein. It is also known as kallikrein III (KLK3), seminin, semenogelase, γ-seminoprotein or P-30 antigen. PSA is the protein in prostate fluid which is responsible for the lysis of the gel proteins, resulting in the liquefaction of the semen[2]. In 1971, PSA was discovered by a Japanese research group as a result from a forensic search to identify specific proteins present in the ejaculate and was originally named γ-seminoprotein[3]. Later, PSA was specifically linked to the prostate and prostatic disease and was shown to be almost exclusively present in the epithelial cells of the prostate[4-6]. PSA was first measured quantitatively in human serum by Papsidero and Kuriyama *et al.* in 1980[7-9].

Normally, PSA is confined within the prostate and only a minute amount leaks into the circulation. In men with PCa, PSA serum levels may be increased. Unlike what might be expected, these elevated serum concentrations in PCa patients are not the result of increased expression of PSA but of an increased release of PSA in the bloodstream, most likely resulting from disruption of the prostate architecture in PCa (Figure 1)[10,11]. As PSA is prostate-specific but not PCa-specific, increased PSA serum levels may also result from prostatitis, irritation or BPH. The clinical usefulness of PSA in PCa detection was first shown by Stamey *et al.* in 1987, who carried out the initial clinical validation[12,13].

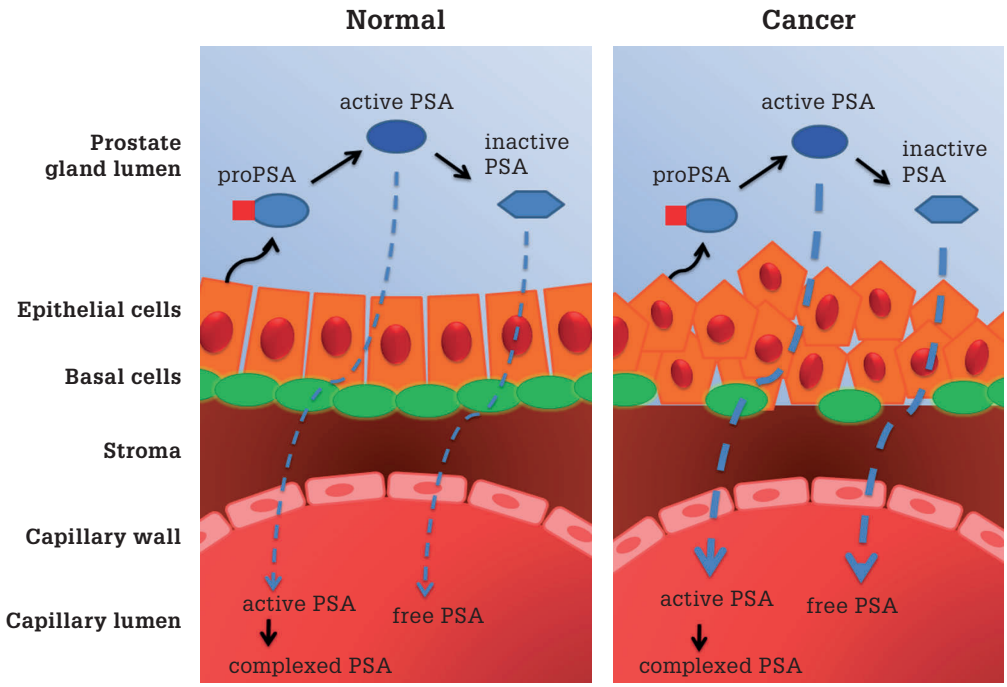

Figure 1. A model of PSA synthesis and secretion. In normal secretory epithelial prostate tissue, proPSA is secreted into the seminal lumen after which active PSA is generated. After proteolysis, active PSA becomes inactive PSA. Minute fractions of both active and inactive PSA diffuse into the peripheral circulation. In PCa, loss of basal cells, disordering of the basement membrane and disruption of normal lumen architecture leads to a decrease in luminal processing and a relative increase of bound PSA and proPSA in the circulation (Figure adapted from reference[10]).

In the circulation, several forms of PSA are present (Figure 1). A substantial fraction of PSA that enters the bloodstream is intact and forms a complex (designated cPSA) with the protease inhibitor α_1-antichymotrypsin or other inhibitors. PSA that is catalytically inactive does not form complexes and circulates as free PSA (fPSA). The major part of fPSA comprises of three distinctive forms; an inactive form (iPSA) similar to active native PSA, a mixture of precursor isoforms of PSA (proPSA or pPSA), and a form designated benign prostatic hyperplasia–associated PSA or 'benign' PSA (BPHA or BPSA), as it was initially found in men with BPH[14]. Total PSA (tPSA) is the sum of the free and complexed forms.

3. Characteristics, diagnosis and screening of prostate cancer

3.1 Characteristics of prostate cancer

Prostate cancer is mainly diagnosed within the elderly male population. As mentioned, PCa mostly originates from the peripheral zone of the prostate. Therefore, it may never cause any symptoms as most prostate tumours are slow-growing, and meanwhile patients die of other causes. However, in a minority of men cancer cells metastasize or progress locally, causing late symptoms such as pain or micturition complaints.

PCa constitutes a major health issue worldwide and is considered as one of the most common causes of cancer-related deaths. The American Cancer Society estimated that in 2013, 238,590 men will be diagnosed with PCa and 29,720 men will die of PCa within the United States[15]. Extrapolated, this means that one out of every six men will be diagnosed with PCa during his lifetime, and one out of every 36 men will die of it. This is in accordance with results from autopsy studies, showing microscopic PCa lesions in 50% of men in their sixth decade, increasing to more than 75% in men older than 85 years[16,17].

Not surprisingly, this poses an enormous burden on total healthcare costs. In 2010, medical costs associated with PCa in the United States alone estimated $12 billion, expected to increase to $16 billion in 2020[18]. In the Netherlands, 168 million euro's were spend on PCa care in 2007, about 0.23% of the total national healthcare budget[19]. For several decades now there has been an extensive search for biomarkers for PCa, in order to detect PCa at an early stage in which curative treatment is still possible. In addition, it is important to distinguish aggressive from indolent disease, hereby limiting overtreatment of PCa.

3.2 The diagnosis of prostate cancer

Before the discovery of PSA, a digital rectal examination (DRE) was the only pre-diagnostic tool to detect PCa. However, often the disease had progressed at time of diagnosis, limiting curative treatment. Originally, tPSA resembled the ideal biomarker for PCa detection. After its introduction and thereafter widespread use in the late 1980s, a dramatic change in the epidemiology of PCa was observed[20]. An enormous increase in the number of newly diagnosed PCa cases was seen, peaking in 1992 and declining thereafter, however not falling back to the level of the pre-PSA era (Figure 2)[21,22]. In addition, it has been suggested that increased PCa awareness as well as improved diagnostic means have also contributed to the increase in PCa incidence during the early 1990s[23].

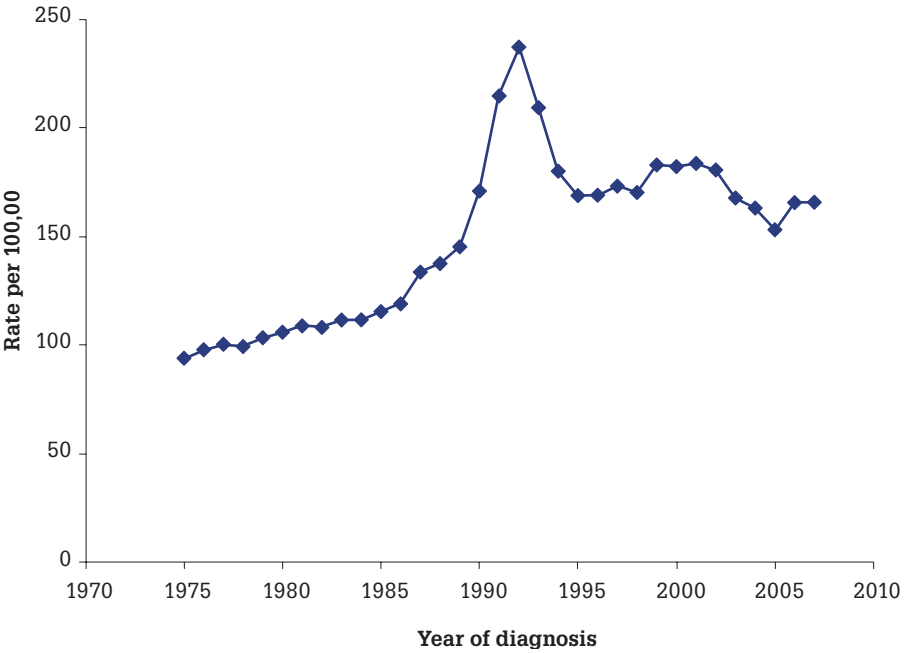

Figure 2. Age-adjusted incidence of PCa (rate per 100,000) by year of diagnosis (all ages) for all races. Data adapted from Surveillance, Epidemiology, and End Results (SEER) Program (www.seer.cancer.gov).

Next to the increased number of detected PCa cases, the widespread use of PSA led to a stage shift, i.e. more cancers were detected at an earlier stage. This is illustrated by the fact that PCa diagnosed by PSA testing has a 70-80% change of being organ-confined, in contrast to only 20-30% before the introduction of PSA[24,25]. Initially, tPSA levels below 4.0 ng/ml were considered more or less as 'normal', or associated with negligible risk of PCa. However, by evaluating prostate biopsies in men with tPSA values below 4.0 ng/ml it became clear that the risk of PCa is certainly not negligible in this patient category[26]. For example, the risk of being diagnosed with PCa at a tPSA level below 2.0 ng/ml is still 17%, including cases of high-grade PCa. By subsequently lowering the threshold limit for prostate biopsies, tPSA theoretically has the capacity to detect the great majority of prostate cancers, though at the cost of greatly increasing the number of false positive outcomes. This, in turn, would lead to an enormous increase in unnecessary biopsies with all the accompanying morbidity. So, tPSA should be regarded as a continuous variable providing a spectrum of PCa risk, rather than focusing on a fixed threshold level, which is set to acquire prostate biopsies or to withdraw from taking action.

In addition, personal risk factors can be added to this spectrum, such as age, ethnicity, family history of PCa, the presence of urinary symptoms and DRE results. Several PCa risk calculators have been developed combining tPSA results and these personal factors, leading indeed to a more accurate calculation of the probability of positive prostate biopsies than by utilizing tPSA alone[27,28]. Other strategies to enhance the diagnostic performance of tPSA include the use of age-specific tPSA cut-offs, tPSA density (tPSA/prostate volume) and tPSA velocity (tPSA over time), showing inconsistent results in increasing the diagnostic accuracy of tPSA[29].

3.3 Prostate cancer screening

Today, many controversies about tPSA-based PCa screening exist. As it is known that most men die with PCa rather than from PCa, tPSA based screening leads to an overwhelming overdiagnosis, often resulting in overtreatment of low-risk PCa patients[30,31]. Several large studies have been performed or are still ongoing to answer the question whether or not screening for PCa actually saves lives and at what costs[32-34]. The rationale for this screening is that by regularly measuring tPSA levels in combination with performing a DRE, PCa might be identified in an early stage in which definitive cure is still possible. However, as PCa is generally a low-grade and slow-growing disease, it takes more than a decade to draw any conclusions whether or not men could actually benefit from this form of screening. The often low-grade and slow-growing nature of PCa also hampers large scale prospective testing and validation of novel promising biomarkers. A recent report of the Prostate, Lung, Colorectal and Ovary (PLCO) screening trial, involving more than 76,000 men between 55 and 74 years, concluded that PCa screening did not result in a decrease in mortality[32]. After thirteen years of follow-up, the cumulative mortality rates from PCa in the screening and control arms were 3.7 and 3.4 deaths per 10,000 person-years, respectively, resulting in a statistically non-significant difference. However, the failure to reach statistical significance might be caused by opportunistic screening in the control arm of this study. In addition, a substantial number of men were pre-screened before randomisation and inclusion. Therefore, it is questionable if this study has sufficient power to demonstrate an effect of screening on PCa mortality[35]. The results of the PLCO screening study are in contrast with the European Randomized Study of Screening for Prostate Cancer (ERSPC). This study involves more than 182,000 men aged 50 to 74 years. After a median follow-up of 11 years the relative reduction in risk of death from PCa in the screening group was 29%. The absolute reduction in mortality in the screening group was 0.10 deaths per 1,000 person-years. To prevent one death from PCa at 11 years of follow-up, 1,055 men would need to be screened and 37 cancers would need to be detected[34]. In contrast to the PLCO study, the number of men pre-screened before

randomisation was estimated to be low and contamination in the control arm was at most 15%, making this study adequately powered to draw conclusions on the effect of screening on PCa mortality. However, based on the conflicting results of these and other screening programs in addition to the large number of men needed to treat for each avoided death, most international and national screening guidelines still recommend against population-based or opportunistic screening for PCa.

Surprisingly, there have not been many studies evaluating the economic consequences of PCa screening. Also from an economic perspective, PCa screening is debatable. Using data from the ERSPC, researchers estimated that $5.2 million would have to be spent on screening to prevent one death from PCa[36]. Extrapolated, this would be $262,758 per life-year saved. However, a recent ERSPC report showed that the number of cancers needed to detect to prevent one death from PCa is decreasing after further follow-up time. So, most likely the costs associated with screening will be lower than the initial estimates, however further studies regarding cost-effectiveness of PCa screening are needed[37].

In conclusion, novel biomarkers are eagerly awaited, as these might help in the more selective identification of men with aggressive forms of PCa, thereby limiting overdiagnosis and overtreatment, and thus limiting overall healthcare costs.

4. Biomarkers for prostate cancer

According to the National Institute of Health, a biomarker is defined as a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention[38]. Specifically related to PCa, these biomarkers could serve four different purposes[39]:

1. Risk: these markers are used to identify men with an increased risk of developing PCa during their lifetime.
2. Diagnostic and screening: these biomarkers can replace classical histopathological characteristics in assessing the presence or absence of PCa and can be preferably implemented in large-scale screening programs to detect PCa at an early stage.
3. Prognostic, predictive and monitoring: these biomarkers are used to predict the outcome of patients diagnosed with PCa, predict the outcome of therapy, and monitor treatment.
4. Surrogate endpoint: these biomarkers are a substitute for a clinical endpoint.

Ideally, a single biomarker would be able to define all characteristics as described above. However, it would be more realistic to suppose that a panel consisting of several biomarkers would be needed to reach such a goal.

4.1 Biomarkers of risk assessment for prostate cancer

It is known that some men have a familial predisposition for developing PCa[40]. Also, it has been shown that African American men have higher PCa incidences and mortality rates compared to European American men, followed by Asian American men and Hispanic men[41,42]. This might be based on certain gene polymorphisms, but might also be due to environmental or behavioural factors. Having a first-degree relative with PCa increases the risk of developing PCa by approximately two- to three-fold[43]. This risk is further increased by early age at onset and multiple relatives with PCa[44,45]. In total, it has been estimated that 5-10% of PCa cases are accounted for by genetic susceptibility[46]. Since the last decades, genetic research has been conducted to identify the polymorphisms and genes responsible for this PCa susceptibility. Genome wide association studies (GWAS) have revealed more than forty germline variants of various genes or chromosomal loci that are significantly associated with PCa susceptibility[47,48]. However, for only a few of these their biological significance and function has been elucidated. In the future, larger GWAS analyses in diverse ethnic groups might provide further genetic insights. These could then be translated into risk estimation and the development of a reliable biomarker for identifying PCa susceptible men.

4.2 Diagnostic and screening biomarkers for prostate cancer

The perfect biomarker (or as mentioned before more likely a panel of biomarkers) would be able to state the diagnosis of PCa at near 100% sensitivity and specificity. Preferably, this marker would also state prognostic information or specifically detect aggressive forms of PCa that need to be treated. If such a biomarker would be available, all PCa cases could be detected with negligible false positive or false negative outcomes. As already extensively described above, the most widely used biomarker to detect PCa is PSA. In addition, PSA forms also the basis of all screening programs to detect PCa population-wide. During the last decades, several isoforms of PSA have been discovered, which have been evaluated in the detection of PCa.

4.2.1 Isoforms of prostate-specific antigen

PSA is produced as a prepro-protein, containing 261 amino acids. After removal of the 17-amino acid leader sequence, an inactive 244-amino acid precursor protein termed proPSA (or pPSA) results (Figure 3)[49].

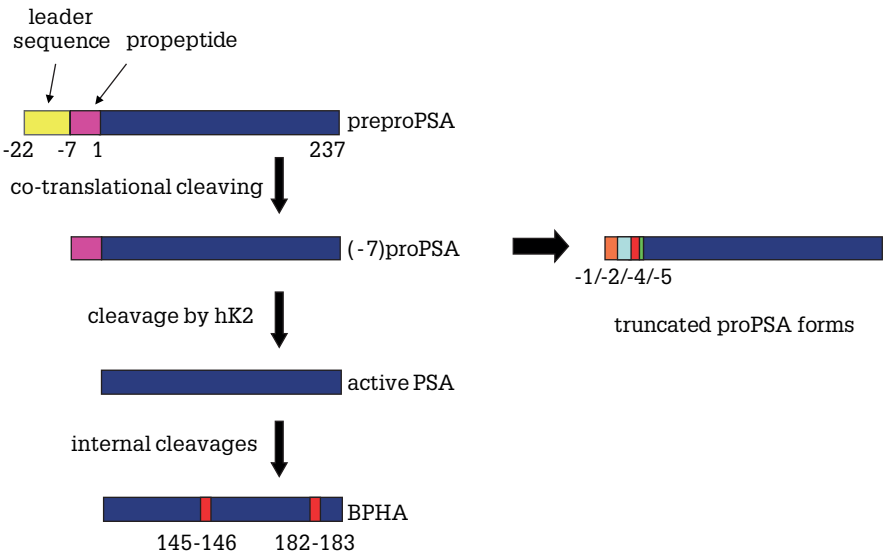

Figure 3. PSA is initially produced as a 261 amino acid prepro-protein. After cotranslational removal of the amino-terminal leader sequence, the non-catalytic zymogen (-7)pPSA results. After cleavage by hK2, the 7 amino acid propeptide is removed and catalytically active mature PSA (237 amino acids) is formed. Alternatively, truncated forms of pPSA are formed due to cleavage within the propeptide. As a result of internal cleavages within active PSA between residues 145-146 and 182-183 the inactive BPHA can be produced (Figure adapted from reference[10]).

After cleavage by human kallikrein 2 (hK2) it is converted to mature, active PSA (237 amino acids)[50,51]. Originally, pPSA was defined as the only precursor form of PSA, consisting of 244 amino acids including a 7 amino-acid pro-peptide leader and was therefore named (-7)proPSA. However, later reports presented several other truncated forms of pPSA, such as (-1), (-2), (-4), and (-5)pPSA, containing 1, 2, 4, or 5 amino acids in the pro-peptide leader, instead of the native 7 amino acids (Figure 3)[52,53].

pPSA was shown to be differentially elevated in peripheral zone cancer and undetectable in most specimens of the transition zone, leading to the assumption that pPSA represented a more cancer-specific form of PSA[54]. Initial attempts to confirm the presence of pPSA in serum were unsuccessful, but later others unequivocally confirmed the presence of several pPSA forms in serum of PCa patients[52,53,55,56]. The molecular basis for the increased serum levels of truncated pPSA forms in PCa patients is unknown, but most likely reflects decreased cleavage

of (-7)proPSA by hK2 in PCa cells. Overall, roughly a third of the fPSA fraction in cancer serum consists of pPSA forms (Figure 4).

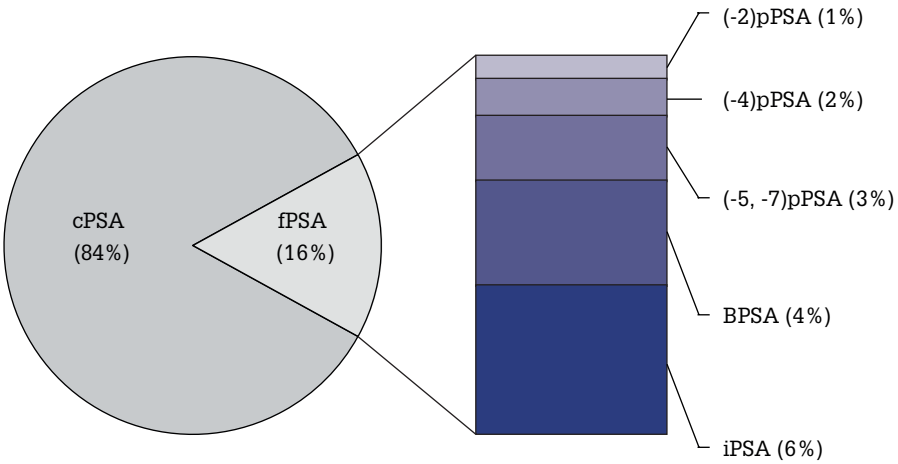

Figure 4. The partitioning of fPSA into various pPSA forms, BPHA and iPSA in PCa serum within the 4-10 ng/ml tPSA range (Figure adapted from reference[57]).

In 2000, Mikolajczyk *et al.* published a report in which PCa tissue was examined to further clarify the origin of pPSA[54]. Sequencing showed that the pPSA in peripheral zone cancer consisted mainly of (-2)pPSA. A subsequent study confirmed the presence of (-2)pPSA in serum of men with PCa, in which (-2)pPSA formed 25-95% of the fPSA fraction, in contrast with 6-19% in biopsy-negative men[52]. As a result of the presence of only a 2 amino acid pro peptide, hK2 was shown to be unable to activate (-2)pPSA to mature PSA, stabilizing (-2)pPSA as an inactive isoform of PSA in serum. Further characterization of fPSA in normal, hyperplastic and cancerous prostatic tissue lead to the identification of a specific molecular form of clipped fPSA, called BPHA (or BPSA), most likely the consequence of posttranslational proteolytic processes within the prostate[58]. It was shown that BPHA contained 237 amino acids, like PSA, but is clipped at amino acid residues lysine 145-146 and lysine 182-183 (Figure 3). %BPHA (BPHA/tPSA) was significantly increased within the transition zone of patients with BPH in comparison to patients without BPH. Initial measurements of BPHA concentrations in serum showed that BPHA represented 25% of the fPSA fraction in biopsy-negative men[59]. Canto *et al.* showed BPHA and fPSA to be strongly correlated with both age and transition zone volume in 91 biopsy-negative men[60]. Furthermore, BPHA outperformed fPSA as well as tPSA in the prediction of transition zone enlargement. A study from Naya *et al.* demonstrated

that BPHA, %BPHA and %fPSA (fPSA/tPSA) performed similarly in the prediction of prostate volume (PV).

In addition to tPSA, the specific value of PSA isoforms in the detection of PCa has been evaluated. Catalona *et al.* measured (-2)pPSA values in a cohort of 1091 serum samples of men with and without PCa within the tPSA ranges of 2-4 ng/ml and 4-10 ng/ml[61]. This two-center study showed that (-2)pPSA levels were higher in PCa patients compared to non-PCa patients, but only in one of the two centres statistical significance was reached within the 2-4 ng/ml tPSA range. The predictive power of %(-2)pPSA (=(-2)pPSA/fPSA) was smaller compared to %pPSA. Mikolajczyk *et al.* later confirmed these results for the 4-10 ng/ml tPSA range in 380 serum samples[62]. However, as Naya *et al.* found no significant difference in (-2)pPSA or %(-2)pPSA levels in men with and without PCa, it remains unclear whether PSA isoforms do possess any additional value in the detection of PCa[63,64]. For BPHA, it was shown that BPHA could not predict the presence of PCa and was not related to tumour volume[63].

4.2.2 Prostate cancer antigen 3 and the TMPRSS2:ERG fusion gene

Next to tPSA, the other commercially available and FDA (US Food and Drug Administration) approved test for the detection of PCa is the differential display clone 3 (DD3) or PCA3 assay (Progensa® PCA3 , Gen-Probe, San Diego, California)[65]. Following a digital rectal examination, PCA3 mRNA can be quantified in urine specimens together with PSA mRNA to generate a so-called PCA3 score. One of the aims of the PCA3 test was to decrease the number of unnecessary prostate biopsies. Several studies have indeed shown a slight increase in diagnostic accuracy over tPSA, although the sensitivity of PCA3 is limited in the tPSA zone of 2-10 ng/ml[38,66-68]. Recently, PCA3 has also been assessed in a screening protocol for PCa. It was shown that PCA3 missed fewer cancers compared to tPSA (32 vs. 65%), but it should be mentioned that this was a tPSA-pre-screened cohort, so results in an unscreened population should be awaited. Alike the tPSA assay, the PCA3 score is also a continuous variable[69]. According to the summary of safety and effectiveness data as published by the FDA, a PCA3 score <25 is associated with a decreased likelihood of a positive prostate biopsy.

Another promising urinary based assay (which is not yet commercially available), is the TMPRSS2:ERG gene fusion test. The TMPRSS2:ERG gene fusion is a PCa-specific genomic rearrangement consisting of a fusion between the strong androgen-regulated TMPRSS2 gene and the proto-oncogene ERG, a member of the ETS transcription factor family. This results in an androgen-regulated TMPRSS2:ERG fusion gene and is found in approximately 50% of all prostate tumours[70].

In 108 PCa patients, urinary sediments were analysed for the presence of fusion transcripts. This resulted in a very high specificity of 93%, in contrast to a limited sensitivity of 37%[71]. A combination of the PCA3 test with the TMPRSS2:ERG assay has shown to further increase diagnostic accuracy. A study performed in 105 men showed that PCA3 alone had an AUC (area under the curve) of 0.65, while the combination of PCA3 and TMPRSS2:ERG increased the AUC to 0.77. This was further increased to 0.80 by adding tPSA to this multivariate model[72].
So, although the addition of PSA isoforms, PCA3 and TMPRSS2:ERG to the tPSA test seems promising, it is clear that more specific markers to identify men with PCa would be of tremendous value.

4.3 Prognostic, predictive and monitoring biomarkers for prostate cancer

4.3.1 Prognostic biomarkers for prostate cancer

Next to diagnosing PCa, an ideal biomarker would also state prognostic information. In this way, indolent disease, for which treatment could be deferred, can be differentiated from aggressive cases of PCa. In addition to the controversies surrounding tPSA in PCa diagnosis, there is an ongoing debate on the role of tPSA as a prognostic factor. It has been shown that higher preoperative tPSA values are associated with increased odds of extracapsular extension, positive surgical margins, seminal vesicle invasion and increased risk of biochemical progression[73]. However, when restricted to tumours identified by needle biopsy because of an elevated tPSA level (<10 ng/ml), pretreatment tPSA levels were only marginally related to biochemical progression. Another study showed that tPSA is an independent predictor of several pathologic stages and may improve the accuracy of multivariate models predicting pathologic stage[74]. Currently, the best predictor for survival at the time of PCa diagnosis is the pathologic grade of the tumour tissue (Gleason score) within the prostate biopsies. A Gleason score (GS) is given based upon the microscopic appearance of the tumour tissue (Figure 5). A first grade is assigned to the most common tumour pattern, and a second grade to the next most common tumour pattern. These two grades together form the GS, which ranges from 2 to 10 (Figure 5). The scoring system is named after Donald Gleason, a pathologist who developed the scoring system in the 1970s[75]. Tumours with a higher GS behave more aggressive and are significantly associated with a worse prognosis[76]. A drawback of the GS grading system is that it is partly subjective, showing significant observer variability[77]. In addition, due to the stage and grade shift of presently detected prostate cancers caused by tPSA screening, the prognostic power of the Gleason scoring system has diminished, as nowadays most patients present with a GS of 6, 7 or 8[78-80].

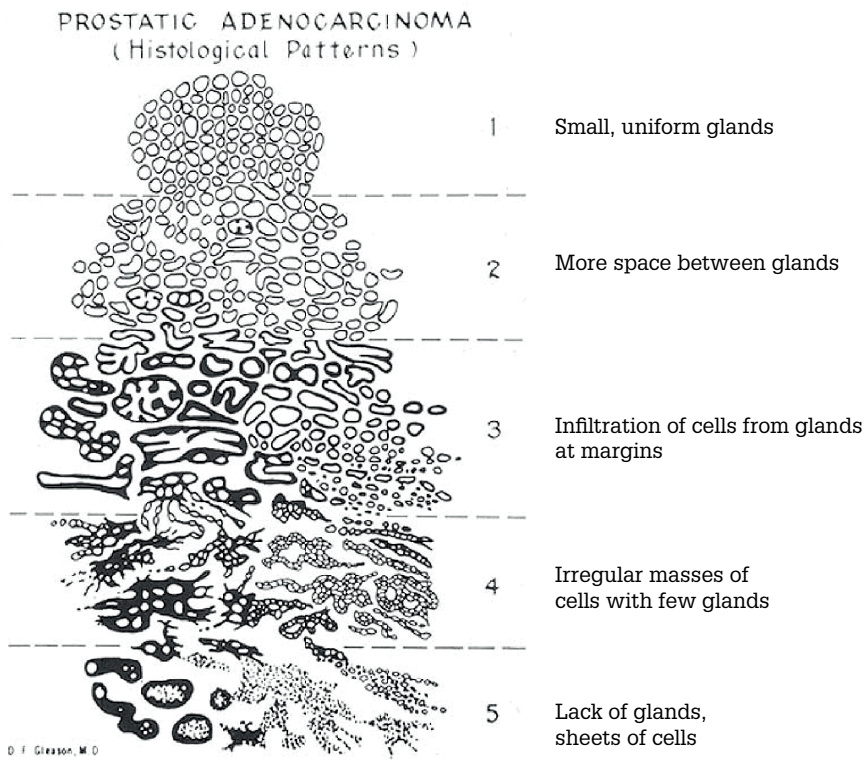

Figure 5. Dr. Gleason's drawing of the five Gleason grades[75].

In the last decades, many alternative prognostic markers for PCa have been proposed, on protein as well as on RNA and genomic level. Examples of alternative markers on protein level are numerous, including various PSA-isoforms. A study by Catalona *et al.* specifically analysed pPSA forms in relationship to PCa aggressiveness[81]. It was shown that (-2)pPSA could not discriminate between cancers confined to the prostate and cancers with extracapsular extension and did not perform significantly better than %pPSA and %fPSA in the detection of cancers with a GS >7. These results were later confirmed by de Vries *et al.* in a smaller study[82]. A recent report by de Vries *et al.* describing 61 men diagnosed with PCa showed that also BPHA failed to discriminate between favourable and poor prognostic PCa[82].

Also the possible role of PCA3 in PCa prognosis has been evaluated, resulting in conflicting outcomes. While some studies found a relationship between PCA3 and small volume tumours and insignificant PCa, others failed to find such a correlation[83]. Furthermore, PCA3 is not significantly associated with locally advanced disease

and it has limited value in the identification of aggressive tumours[83]. With regard to the TMPRSS2:ERG gene fusion, no significant relationship was found between the presence of the fusion transcripts and Gleason scores in prostate biopsies[38,71,84]. In addition, data on the association of TMPRSS2:ERG and patient outcome are conflicting[85]. Other markers that have been evaluated are PSCA (prostate stem cell antigen), hK2 (human kallikrein 2), EPCA (early prostate cancer antigen) and AMACR (alpha-methylacyl CoA racemase), but none of these has entered into routine clinical practice yet[86-90]. Currently, the best and independent pretreatment prognostic marker is the pathologic grade of the tumour tissue within the prostate biopsies, so prognostic blood or urine based biomarkers are urgently awaited[76].

4.3.2 Monitoring and predictive biomarkers for prostate cancer

Regarding the monitoring of men treated for PCa with curative intent, either by radiation therapy (RT) or radical prostatectomy (RP), the use of tPSA has been extensively studied. After a successful RP, all prostatic tissue is removed and tPSA levels should become undetectable. Hence, detectable serum tPSA levels are indicative of residual prostatic tissue, which presumably represents locoregional or systemic cancer[91]. The American Urological Association (AUA) has defined a biochemical recurrence as a serum tPSA level ≥0.2 ng/ml, confirmed by a second determination, after initial undetectable tPSA levels following RP[92]. The kinetics of tPSA increment after RP are indicative of the location of tumour recurrence. If tPSA never falls to undetectable levels or is rising rapidly after RP, systemic disease is more likely than residual disease[93,94]. In contrast, if tPSA levels rise slowly after having been undetectable for a longer period of time, isolated local recurrence in the prostatic bed is more likely[93,95]. In this way, tPSA can be used to guide adjuvant therapy after RP.

After external RT, brachytherapy (internal RT) or cryotherapy (freezing of prostate tissue) tPSA may still be detectable, as the prostate gland is not removed. This makes it more difficult to define biochemical recurrence compared to RP. The decline in tPSA after RT is gradual and the mean time for tPSA to reach its nadir (the lowest level to which tPSA drops) is 18 months or longer[96]. This nadir is a strong indicator of treatment success, however, the rate of decline does not correlate to the risk of PCa relapse[96-99]. According to the latest definition, a tPSA level higher than 2 ng/ml above the nadir is considered as a biochemical failure after RT[100]. A complicated issue is the phenomenon of a tPSA bounce after RT, typically occurring after 12 to 18 months[101]. This can occur in the absence of disease and does not necessarily signify treatment failure. Up to date, there are no definitive methods or markers to distinguish a tPSA bounce from cancer recurrence. So, novel markers distinguishing the latter two conditions could have additional value in guiding patient therapy.

4.4 Surrogate endpoint biomarkers for prostate cancer

As PCa is usually a low aggressive and slow-growing disease, trials and studies involving PCa are often of extensive duration. Therefore true endpoints, such as mortality from PCa, are often replaced by surrogate endpoints such as tPSA. An extensive review on tPSA as a surrogate endpoint marker for PCa concluded that tPSA is not a suitable replacement for survival, especially when second or third line treatments become more efficacious[102]. This is further illustrated by the fact that prognostic studies have shown that in hormone independent disease only 17% of survival is explained by time-dependent tPSA measurements, showing sufficient variation in survival unaccounted for by tPSA measurements[103]. Therefore, also from the viewpoint of surrogate endpoints, novel markers are currently awaited.

5. Challenges in biomarker research

5.1 Biomarker research phases

The search for novel biomarkers for PCa can be divided into three distinctive phases. First, there is a discovery phase, resulting in the identification of a number of promising candidate markers. Then, during the verification phase, the list of potential candidates is narrowed, allowing only the most promising candidates to enter the validation phase, which should ultimately lead to the clinical implementation of a novel biomarker. All these three phases have their own difficulties and challenges.

5.2 Selection of biological specimen

When searching for a novel biomarker, the first question that should be addressed is which body fluid should be utilized. Preferably, this body fluid should be accessible in a non or minimally invasive way. So, the two body fluids that would be most appropriate are urine or blood. The drawback of utilizing the first one is that there are currently no biobanks available containing large numbers of urine samples with long-term follow-up, so large scale validation of novel markers is seriously hampered. Due to the slow-growing and low-grade characteristics of the majority of PCa cases, it would take up to a decade to draw any conclusions from a prospective urine-based study, especially from a prognostic point of view. So, at least for now, this favours blood (serum or plasma) as the more suitable of the two. In addition, as physicians are so familiar to PSA-based decision making, it is highly unlikely that a novel biomarker will replace PSA, meaning blood will we be drawn anyway.

5.3 Selection of cellular material

The second question that should be addressed is which cellular material should be explored to look for novel markers. In short, this could be on metabolite, protein, RNA or DNA level. As proteins produced by cancer cells (or their microenvironment) may eventually enter the circulation, these could be directly assessed in the serum or plasma of men suspected of having PCa. This is the case for most successful tumour markers applied in the clinic today, such as alpha fetoprotein (AFP), beta human chorionic gonadotropin (β-hCG), cancer antigen 125 (CA125), and of course PSA.

Another advantage of a protein biomarker would be that standard detection techniques could be utilized, such as enzyme-linked immunosorbent assays (ELISA), facilitating incorporation into current clinical laboratory practice.

Next to protein markers, novel biomarkers could also be found on RNA and DNA levels, as genetic and epigenetic alterations are common events in PCa. In contrast to protein markers, these are usually detected in tissue samples, requiring invasive methods to obtain the tissue itself. Another option is the detection of RNA and DNA directly in serum or in circulating tumour cells, but this requires more elaborate techniques, currently not standard in clinical laboratory practice. However, recent publications have shown that RNA profiling of whole blood samples is a feasible technique which might yield prognostic information in PCa[104,105].

5.4 Mass spectrometry for biomarker identification

One approach to discover novel biomarkers is by utilizing comparative mass spectrometry (MS), analysing human serum samples in search of tumour-specific proteins shed into the circulation by tumour cells. MS is an analytical chemistry technique based on the determination of the mass of an analyte ion. This analyte can be fragmented inside the mass spectrometer to give charged product ions. The masses of the fragment ions can then be determined in a second stage of MS, giving rise to the term MS/MS or tandem mass spectrometry. Improved specificity can be obtained by monitoring a precursor ion and one of its collision-induced dissociation-generated product ions, a technique known as multiple reaction monitoring (MRM). This technique also reduces the possibility of a false-positive identification and inaccurate quantitation. As well, MRM analysis can be multiplexed, allowing the quantitation of hundreds or even thousands of target molecules in a single analysis. In addition, a recent multi-laboratory analysis showed that MRM results are reproducible between different laboratories[106].

To identify specific proteins, a bottom-up approach can be used in which a proteolytic enzyme, typically trypsin, is used to degrade proteins into smaller peptides. Subsequently, these peptides can be introduced into the mass spectrometer and

identified. The identified masses can be used as input for a database search of predicted masses that would arise from digestion of a list of known proteins. If a protein sequence in the reference list gives rise to a significant number of predicted masses, there is evidence that this protein is present in the original sample.
However, serum is a highly complex fluid with a high variability between human serum samples. It has a vast dynamic range in protein concentrations (>10^{10}) and dominance of a small number of high abundant proteins, such as albumin and immunoglobulins, which constitute more than 99% of the entire protein content, masking the presence of low abundant proteins, including possible novel biomarkers[107].
To rule out these variables, hundreds to thousands of samples have to be analysed in order to identify discriminating proteins. In addition, current technologies, capable of analyzing protein concentrations spanning four orders of magnitude, would be missing the lower abundant proteins (such as PSA) which are likely to constitute the next generation of biomarkers.

1. Assay development and validation
A clinical assay is developed in this phase. The assay must discriminate individuals with cancer from those without. The patients assessed in this phase have established disease. The utility of the assay in detecting disease early is not demonstrated in this phase.
2. Retrospective longitudinal clinical repository studies
Specimens collected and stored from a cohort of healthy individuals who were monitored for development of cancer are used here. Evidence for the capacity of the biomarker to detect preclinical disease is demonstrated in phase 2.
3. Prospective screening studies
In this phase, individuals are screened with the assay and diagnostic procedures are applied to those who screened positive. This can help to establish the tumor stage or the nature of the disease at the time of detection.
4. Randomized control trials
The objective of this phase is to determine if screening reduces the burden of cancer in the population.

Figure 6. The phases of biomarker validation (adapted from reference[109]).

5.5 Biomarker validation issues

After preclinical exploratory studies, in which promising biomarker candidates are identified, these should be enrolled in extensive validation programmes. This is necessary, as methods of marker discovery, study design, patient selection,

interpatient heterogeneity and intratumour heterogeneity are all confounding factors, especially in the case of PCa. Other important confounding factors are the danger of bias, the risk of overfitting of data and issues related to the handling and storage of clinical specimens[108]. Several phases can be identified in the process of biomarker validation, as depicted in Figure 6[109]. Ideally, all phases should be successfully completed before a biomarker enters clinical practice.

5.6 Biomarker standardisation issues

Another issue related to the validation of a biomarker is its standardisation or calibration. Preferably, before a biomarker enters clinical practice, identical calibration protocols and standards should be used, resulting in identical diagnostic performances amongst different assay platforms. Unfortunately, this is only partly the case for tPSA.

The number of ordered tPSA tests has increased dramatically during the last decade. In the Netherlands, almost 28% of men older than 65 years had their tPSA tested in 2001, increasing to over 45% in 2011[110]. As these tPSA tests are performed on various assay platforms, standardisation is of utmost importance to improve clinical interchangeability and to compare long-term results. Today, more than 30 different types of tPSA assay reagent sets are available[111]. In the past, several publications have shown that this wide variety of tPSA assays leads to significantly different values for tPSA. Possible causes of this lack of interchangeability are differences in assay design, differential epitope recognition, cross-reactivity to tPSA homologous antigens and the lack of equimolarity[5,112-115].

As most clinicians are unaware of their local assay used to measure tPSA levels, misinterpretation of tPSA values will occur, for example influencing the decision to recommend that a patient undergoes a prostate biopsy for PCa detection[113]. To improve the interchangeability of tPSA assays, the First International Standard for tPSA was introduced in 1992 and later adopted by the World Health Organization (WHO)[116,117]. This indeed decreased differences between the various tPSA assays[118-120]. However, for certain tPSA assays, this restandardisation caused a shift in mass units, resulting in a potential under or over-recovery of tPSA values. Consequently, if this shift is not fully appreciated around the threshold for prostate biopsies, a change in PCa detection rates may result with possible life-altering consequences for individual patients.

6. Scope of the thesis

This thesis focuses on the full spectrum of PCa biomarkers, ranging from the discovery and (pre-) validation of novel markers, to assessing the clinical value of recently discovered PSA isoforms and the implications of the introduction of a tPSA calibration standard to decrease interassay variability.

In order to specifically detect proteins secreted into the circulation by PCa cells, we developed a xenograft model system (Chapter 2). In this system, human prostate cancer cell lines are transplanted onto athymic nude mice. We hypothesized that after successful inoculation, PCa cells would shed proteins into the mouse circulation, mimicking the situation in humans. Thus, a normal nude mouse would only have mouse proteins in its circulation, while the serum of a nude mouse bearing a human xenograft would also contain human proteins in addition to mouse proteins. In order to identify these tumour-derived proteins, the serum of normal and xenografted mice was analysed by mass spectrometry.

In Chapter 3, this xenograft model system was further exploited by utilizing the mouse immune system. We hypothesized that by immunizing immune-competent congenic mice with serum drawn from nude mice grafted with human prostate cancers, an immune response would occur, generating immunoglobulins specifically directed against PCa-derived proteins. Subsequently, protein microarrays were utilized to profile the antibody production and indirectly identify PCa xenograft-derived antigens.

In Chapter 4, we analysed the diagnostic and prognostic performances of the PSA isoforms BPHA and p2PSA in a large multicenter study. We hypothesized that these isoforms, as well as its derivative *phi* (Beckman Coulter Inc. name for the "prostate health index," a mathematical combination of PSA, fPSA and p2PSA, see www.prostatehealthindex.org), would have significant additional value in PCa detection, next to tPSA and fPSA. Secondly, we hypothesized that these PSA isoforms could have additional value in discriminating aggressive from indolent disease and thereby aid in PCa prognosis.

Another essential step in the establishment of a biomarker is its international calibration or standardisation. In Chapter 5, we focused on the consequences of the implementation of an international calibration standard for tPSA assays, specifically for the Beckman Coulter tPSA assay. We hypothesized that the implementation of this international calibration standard would lead to a significant shift in tPSA results, thereby directly influencing PCa detection rates.


CHAPTER 2

EXOSOMAL SECRETION OF CYTOPLASMIC PROSTATE CANCER XENOGRAFT-DERIVED PROTEINS

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Abstract

Novel markers for prostate cancer (PCa) are needed because current established markers such as prostate-specific antigen lack diagnostic specificity and prognostic value. Proteomics analysis of serum from mice grafted with human PCa xenografts resulted in the identification of 44 tumour-derived proteins. Besides secreted proteins we identified several cytoplasmic proteins, among which were most subunits of the proteasome. Native gel electrophoresis and sandwich ELISA showed that these subunits are present as proteasome complexes in the serum from xenograft-bearing mice. We hypothesized that the presence of proteasome subunits and other cytoplasmic proteins in serum of xenografted mice could be explained by the secretion of small vesicles by cancer cells, so-called exosomes. Therefore, mass spectrometry and Western blotting analyses of the protein content of exosomes isolated from PCa cell lines was performed. This resulted in the identification of mainly cytoplasmic proteins of which several had previously been identified in the serum of xenografted mice, including proteasome subunits. The isolated exosomes also contained RNA, including the gene fusion TMPRSS2:ERG product. These observations suggest that although their function is not clearly defined cancer-derived exosomes offer possibilities for the identification of novel biomarkers for PCa.

Introduction

For several decades now, prostate-specific antigen (PSA) has been utilized as the "gold standard" biomarker for the detection of prostate cancer (PCa)[13]. Its introduction caused a dramatic decrease in the prevalence of advanced stages of PCa[20]. However, ongoing efforts are being made to discover new biomarkers for PCa because it became clear that PSA has limited diagnostic specificity and prognostic value, leading to an enormous increase in unnecessary biopsies and overtreatment of low risk PCa patients[26]. In the last decades, many alternative diagnostic or prognostic markers for PCa have been proposed on protein as well as on RNA and genomic levels. Examples of alternative markers on the protein level are numerous, including various PSA isoforms, prostate stem cell antigen, human kallikrein 2, early prostate cancer antigen, and α-methylacyl-CoA racemase[86-90]. On the RNA level, the PCA3 test and especially the recently discovered fusion of TMPRSS2 with ETS transcription factors may hold promise for PCa detection and potentially prognosis in the near future[121,122]. One of the drawbacks of the latter two as markers for PCa is the fact that they are detected in urine, after a standardized prostatic massage, instead of in serum or plasma. This will hamper retrospective validation

as most historical biorepositories do not contain urine. Although several validation studies of promising candidates have been performed in the past or are currently underway, no single marker has yet outperformed PSA, justifying ongoing efforts in searching for PCa biomarkers. One approach is the screening of large series of serum samples from men with and without PCa. However, given the large sample variability, the high complexity, and dynamic range of proteins in serum samples, large numbers of human serum samples have to be analysed to achieve any statistical significance. Also identified proteins may be related to secondary body defence mechanisms rather than being directly derived from the tumour cells as are most tumour markers applied in the clinic today. To circumvent these problems, we have exploited the xenograft model system as a platform for the discovery of new biomarkers for PCa[123]. As has recently been reported, this model system is indeed capable of identifying human proteins that are shed into the circulation by human prostate cancer cells[124]. In the present study we further exploited this approach and performed an in-depth proteomics analysis of serum of mice carrying androgen-sensitive (PC346) or androgen-independent prostate cancer xenografts (PC339). Among the discovered human proteins were numerous cytoplasmic proteins, such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), lactate dehydrogenases A and B, and various subunits of the proteolytic proteasome complex[124]. Many of these cytoplasmic proteins are also present in the human plasma proteome as retrieved from the database of the Human Proteome Organisation Plasma Proteome Project[125]. We hypothesized that the presence of cytoplasmic tumour-derived proteins in the xenograft sera could be explained by the secretion of exosomes. Exosomes are small membrane vesicles secreted by virtually every cell type, including tumour cells[126]. Exosomes are formed in multivesicular bodies by inward budding, thereby encapsulating cytoplasmic components[126,127]. The exact function of exosomes in tumour cells has yet to be elucidated but is expected to relate to roles in cell-to-cell contact, tumour-stroma interaction, protein degradation, and antigen presentation[126,127]. In addition to containing proteins, it was recently discovered that exosomes also contain functional RNA, proposed as "exosomal shuttle RNA"[128]. To confirm our hypothesis that the cytoplasmic tumour-derived proteins in the serum of xenograft-bearing mice were the result of exosomal secretion, we isolated exosomes from the PC346C cell line and analysed their protein content. To further explore the contents of exosomes we isolated and analysed exosomal RNA from both the PC346C and VCaP cell lines.

Experimental procedures

Xenograft serum collection

Human prostate cancer xenografts were grown on immune-incompetent mice athymic male nude (nu/nu) BALB/c mice (n=9 for each xenograft; Taconic, Ry, Denmark)[123,124]. We used the human prostate cancer cell lines PC346 (androgen-sensitive) and PC339 (androgen-independent). Specific characteristics have been described previously[129]. Prior control serum was collected by retro-orbital punction. Tumour-bearing mice were sacrificed after 4–5 weeks, and blood was collected. Samples were stored at -80 °C. The protocol was approved by the Animal Experiments Committee under the national Experiments on Animals Act and adhered to the rules laid down in this national law that serves the implementation of "Guidelines on the protection of experimental animals" by the council of Europe under Directive 86/609/EC.

Preparation of xenograft sera for mass spectrometry

After filtration using a 0.22-µm spin filter, high abundance proteins were removed utilizing Multi Affinity Removal Spin cartridges (Agilent Technologies, Wilmington, DE) according to the manufacturer's instructions. Depleted samples were concentrated on 5-kDa-cutoff ultracentrifugation columns (Agilent Technologies). Total protein concentration was determined by the Bradford method (Bio-Rad). Precast 4–20% polyacrylamide linear gradient gels (Bio-Rad) were utilized to separate 10 µg of protein of depleted mouse serum (pooled from nine individual control mice, nine PC339 xenograft-bearing mice, or nine PC346 xenograft-bearing mice) by SDS-PAGE (Mini-Protean III, Bio-Rad). Prestained high range molecular weight markers (See- Blue, Invitrogen) were loaded on each gel. After running, gels were stained by Coomassie Brilliant Blue (Merck). Gel lanes (range, 5–200 kDa) were excised and divided into 3-mm sections. Gel slices were washed, destained twice (50% (v/v) acetonitrile in 50 mM ammonium bicarbonate), dehydrated (100% acetonitrile), and reduced with 6.5 mM DTT in 50 mM ammonium bicarbonate for 1 h at 37 °C. After alkylation with 54 mM iodoacetamide in 50 mM ammonium bicarbonate, proteins were dehydrated in 100% acetonitrile and then rehydrated with the digestion solution containing 10 ng/l ultra grade sequencing trypsin (Promega, Madison, WI) for 30 min at room temperature. After addition of 30 µl of 50 mM ammonium bicarbonate solution, gel particles were incubated overnight at 37 °C. The peptides were extracted using 0.5% formic acid in 50% acetonitrile, dried completely in a vacuum centrifuge, and stored at 80 °C until analysis.

Liquid chromatography-mass spectrometry of xenograft sera

Nanoflow LC-tandem mass spectrometry was performed for samples by coupling an Agilent 1100 HPLC system (Agilent Technologies), operated as described previously[124], to a 7-tesla LTQ-FT mass spectrometer (FT-ICR-MS, Thermo Electron, Bremen, Germany). For protein identification, database searches were performed using Mascot version 2.0 (Matrix Science, London, UK) allowing 5-ppm mass deviation for the precursor ion, a 0.6-Da tolerance on the fragment ions, and trypsin as the digestion enzyme. A maximum number of one missed cleavage was allowed, and carbamidomethylated cysteine and oxidized methionine were set as fixed and optional modifications, respectively. Only peptides with Mascot scores >30 were accepted. Scaffold (version 01_05_06, Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 90.0% probability as specified by the Peptide Prophet algorithm[130]. Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm[131]. Before we annotated a certain peptide derived from the xenograft-bearing mice as human, a stringent selection procedure was followed (see Figure 1). First all peptide mass values identified in the serum from control mice and PC339 or PC346 xenograft-bearing mice were searched against both the International Protein Index (IPI) mouse and IPI human databases (version 3.18, containing 53,788 and 60,090 proteins, respectively). Then a selection was made of peptides uniquely present in the serum of PC346 or PC339 xenograft-bearing mice. These peptides were subsequently divided into a group of human-specific peptides (identified only in the IPI human database) and a group of homologous peptides (present in both the IPI human and IPI mouse databases). Homologous peptides were annotated as tumour-derived if four or more times higher abundant in the serum of PC339 or PC346 xenografted mice in comparison with control serum as listed in Scaffold. Additionally to double check human specificity, the identified human-specific peptides were blasted against the Swiss-Prot database of the National Center for Biotechnology Information (NCBI) database.

Two-dimensional SDS-PAGE analysis of proteasomes

To clean up samples from contaminants, for each xenograft-derived serum sample (50 µg of protein) the 2-D Clean-Up kit (Amersham Biosciences) was utilized according to the manufacturer's instructions. Next samples were solubilised in 125 µl of rehydration buffer (8 M urea, 2% CHAPS, 0.5% IPG buffer, 0.2% DTT, trace of bromphenol blue, all dissolved in H2O). The samples were loaded onto Immobiline dry strip gels (pH 3–10, non-linear, 7 cm; Amersham Biosciences). Isoelectric focusing

was carried out as follows: 30 V for 10 h, 300 V for 2 h, 1000 V for 30 min, 5000 V for 90 min, 5000 V for 30 min, and 20 V for 20 h. Before starting the second dimension, strips were reduced and alkylated for 15 min in DTT equilibration buffer (6 M urea, 50 mM Tris, pH 8.8, 20% glycerol, 2% SDS, 1% DTT) and iodoacetamide equilibration buffer (6 M urea, 50 mM Tris, pH 8.8, 20% glycerol, 2% SDS, 2.5% iodoacetamide). Next the IPG strips were placed upon a Criterion XT bis-Tris gel (12%; Bio-Rad). The second dimension was run at 100 V for ±2 h with XT MOPS buffer (Bio-Rad). After running the second dimension, gels were blotted onto Protran nitrocellulose membrane in Tris-glycine-SDS buffer (Bio-Rad). The immunoblot was blocked for 1 h and after washing twice incubated overnight at 4 °C with a monoclonal antibody (1:2000) against proteasome subunits 6, 2, 4, 5, 1, and 3 (clone MCP231, Biomol International, Exeter, UK). This corresponds with the subunits 1, 2, 3, 5, 6, and 7 according to the nomenclature of Baumeister *et al.*[132]. In addition, monoclonal antibodies specifically directed against the proteasome 1 subunit (PSMA1; 6 according to the Baumeister *et al.*[132] nomenclature) (clone MCP20, Biomol International) or 3 subunits (PSMA3; 7 according to the Baumeister *et al.*[132] nomenclature) (clone MCP72, Biomol International) were utilized. The immunoblot was washed and incubated for 1 h with a 1:1000 solution of a goat anti-mouse horseradish peroxidase-conjugated antibody (DakoCytomation, Glostrup, Denmark). The secondary antibody was visualized with a chemiluminescence detection kit (Roche Applied Science). For reprobing, blots were immersed in a 0.04 M Tris-HCl, 0.06 M Tris base, 0.07 M SDS, 0.10 M β-mercaptoethanol solution for 20 min at 50 °C.

Native gel electrophoresis of proteasomes

The protocol for characterization of the proteasome by native gel electrophoresis was followed as previously described by Elsasser *et al.*[133]. Depleted xenograft and control serum samples were mixed with 5sample buffer containing 250 mM Tris-HCl, pH 7.4, 50% glycerol, 60 ng/ml xylene cyanol. Samples were either directly loaded or denatured by heating at 96 °C for 5 min. Gels were run for 3–4 h at 4 °C. Gels were transferred onto Protran nitrocellulose membranes at 250 mA for 1.5 h.

Sandwich ELISA for quantification of the proteasome

Serum proteasome concentrations were measured as previously described by Dutaud *et al.*[134] with some minor modifications. Briefly serum from control (n=3) and PC339 (n=3) or PC346 (n=3) xenograft-bearing mice (1:20 diluted) was incubated for 1 h on a plate coated with a 1:4500 dilution of a monoclonal antibody against PSMA1 (clone MCP20, Biomol International). After addition of a 1:1500 solution of a rabbit anti-proteasome antibody (directed against β subunits of the proteasome; PW 8155, Biomol International) cells were extensively washed with PBS-Tween 20 buffer.

Then a 1:4000 solution of goat anti-rabbit horseradish peroxidase-conjugated antibody (DakoCytomation) was added, and the plate was incubated for 1 h in the dark. To reveal horseradish peroxidase activity, 50 mM phosphate, 25 mM citrate buffer, pH 5.0 was added to the cells. After 15 min, the reaction was stopped with 2.5 M sulfuric acid. Absorbance values were measured at 492 nm. All analyses were performed in triplicate.

Cell culture and isolation of PC346C and VCaP-derived exosomes

The human prostate cancer cell line PC346C was cultured in Dulbecco's modified Eagle's medium-Ham's F-12 medium (Cambrex Bio Science, Verviers, Belgium) supplemented with 0.1 nM R1881, 2% FCS (PAN Biotech, Aidenbach, Germany), 1% insulin-transferrin-selenium (Invitrogen), 0.01% BSA (Roche Applied Science), 10 ng/ml epidermal growth factor (Sigma-Aldrich), 100 units/ml penicillin and 100 g/ml streptomycin antibiotics (Cambrex Bio Science), 100 ng/ml fibronectin (Harbor Bio-Products, Tebu-bio, the Netherlands), 20 g/ml fetuin (ICN Biomedicals, Zoetermeer, The Netherlands), 50 ng/ml cholera toxin (Sigma-Aldrich), 0.1 mM phosphoethanolamine (Sigma-Aldrich), and 0.6 ng/ml triiodothyronine (Sigma-Aldrich)[129]. The human PCa cell line VCaP was cultured in RPMI 1640 medium (Cambrex Bio Science) supplemented with 10% dextran-coated charcoal-treated FCS (PAN Biotech) and 100 units/ml penicillin and 100 g/ml streptomycin antibiotics (Cambrex Bio Science). Exosomes were isolated according to the protocol described previously by Hegmans *et al*.[135]. Briefly PC346C and VCaP were cultured in their respective medium to 80% confluency. Cultures were washed twice with PBS and incubated for 48 h in a humidified atmosphere of 5% CO2, 95% air with serum-free medium consisting of Dulbecco's modified Eagle's medium-Ham's F-12 or RPMI 1640 medium (Cambrex Bio Science) supplemented with 0.1 nM R1881. After incubation cell culture supernatants were subjected to successive centrifugations of 400 g (10 min), 3000 g (20 min), and 10,000 g (30 min). Exosomes were then pelleted at 64,000 g for 110 min using an SW28 rotor (Beckman Coulter Instruments, Fullerton, CA). Exosome pellets were resuspended in 0.32 M sucrose and centrifuged at 100,000 g for 1 h (SW60 rotor, Beckman Coulter Instruments). For several experiments, the isolated exosomes from PC346C were further purified by immobilization onto magnetic beads. In short, 25 µl of Dynabeads, precoated with goat anti-mouse immune globulin G (Invitrogen Dynal AS, Oslo, Norway) were incubated for 1 h with 30 µl of an anti-CD9 monoclonal antibody (clone MM2/57, Chemicon International, London, UK). Thereafter beads were incubated by rotation top end over with 20 µg of exosomes for 1 h at 4 °C. After washing four times, beads and exosomes were resuspended in PBS for further experiments.

Electron microscopy of isolated exosomes

Exosomes from PC346C obtained after ultracentrifugation of cell culture supernatants were resuspended in 10 µl of Milli-Q and spotted onto Formvarcoated grids (200 mesh). Adsorbed exosomes were fixed in 2% paraformaldehyde for 5 min at room temperature. After fixation the exosomes were either directly negatively stained using uranyl acetate or immunolabeled with antibodies against CD9 (clone MM2/57, Chemicon International). Antigen-antibody complexes were visualized with protein A conjugated with 10-nm colloidal gold particles (1:20 dilution; Aurion, Wageningen, The Netherlands) followed by negative staining (see above). The specificity of the labelling procedure was tested by omitting the primary antibody. Grids were examined by a Philips CM100 electron microscope at 80 kV.

Mass spectrometry of exosomes

After resuspending the exosome pellet in PBS, 10 µg of isolated exosomes and 10 µg of supernatant fraction were applied onto two 10% SDS-polyacrylamide gels. After running, one of the gels was silver-stained as described previously by Mortz *et al.*[136]. This gel was used to identify distinct bands present in the exosome fraction (see Figure 4b). Subsequently these bands were excised from a Coomassie Brilliant Blue (Merck)- stained gel and cut in 3-mm sections. Preparation for mass spectrometry was performed using the protocol described under "Preparation of Xenograft Sera for Mass Spectrometry". Peptide separation was performed on a nanoscale liquid chromatography system (nanoLC Ultimate 3000) (Dionex, Sunnyvale, CA) with a 50-min gradient (5–40% acetonitrile, H2O, 0.1% formic acid). The injection volume was 5 µl of the tryptically digested sample. Peptides were separated on a C18 PepMap column (150 mm 75 µm inner diameter) (Dionex) at 200 nl/min after preconcentration on a trap column (1 mm 300 µm inner diameter). Separated peptides were detected by a linear ion trap Orbitrap (LTQ-Orbitrap) mass spectrometer (Finnigan LTQ Orbitrap XL, Thermo Electron). Samples were measured in a data-dependent acquisition mode. In the measurement method used, the peptide masses are measured in a survey scan at a maximum resolution of 60,000. To obtain a maximum mass accuracy a prescan is used to keep the ion population in the Orbitrap for each scan approximately the same. During the high resolution scan in the Orbitrap the five most intense monoisotopic peaks in the spectra were fragmented and measured in the LTQ. The fragment ion masses were measured in the LTQ to have a maximum sensitivity and a maximum amount of MS/MS data. For a full analysis of the exosomal proteome, 10 µg of the isolated exosome fraction was applied onto a 10% SDS-polyacrylamide gel and run for 1.5 cm inside the running gel. Thereafter this gel section was excised and divided into 3-mm sections, washed, destained (100% acetonitrile followed by 50 mM ammonium bicarbonate),

dehydrated (100% acetonitrile), and reduced with 6.5 mM DTT in 50 mM ammonium bicarbonate for 45 min at 60 °C. After alkylation with 54 mM iodoacetamide in 50 mM ammonium bicarbonate, proteins were dehydrated in 100% acetonitrile and then rehydrated with the digestion solution containing 10 ng/l ultra grade sequencing trypsin (Promega) for 30 min on ice. After removal of the redundant trypsin solution and addition of 50 mM ammonium bicarbonate solution to cover the gel pieces, gel particles were incubated overnight at 37 °C. After extraction, the peptides were dissolved in 5% formic acid and stored at -80 °C until analysis. Mass spectrometry was performed using the protocol described under "Liquid Chromatography-Mass Spectrometry of Xenograft Sera." For protein identification, database searches were performed using Mascot version 2.2 (Matrix Science) allowing 5-ppm mass deviation for the precursor ion, a 0.6-Da tolerance on the fragment ions, and trypsin as the digestion enzyme. A maximum number of one missed cleavage was allowed, and carbamidomethylated cysteine and oxidized methionine were set as fixed and optional modifications, respectively. All peptide mass values identified in the isolated exosomes were searched against the IPI human database (version 3.37, containing 69,164 proteins). Only peptides with Mascot scores >30 were accepted. Scaffold (version 2_01_02, Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 90.0% probability as specified by the Peptide Prophet algorithm[130]. Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm[131].

One-dimensional SDS-PAGE analysis and western blotting

For one-dimensional electrophoresis, samples containing 10 µg of protein were mixed with Laemmli sample buffer (1:1 ratio) and loaded onto 10% SDS-polyacrylamide gels. Gels were transferred onto a Protran nitrocellulose membrane for Western blotting. The following antibodies were used: CD9 (1:500 dilution; clone MM2/57, Chemicon International), RAB5A (1:200 dilution; clone FL-215, Santa Cruz Biotechnology, Santa Cruz, CA), RAB11A (1:100 dilution; Invitrogen), hepatocyte growth factor-regulated tyrosine kinase substrate (HGS; previously known as HRS; 1:500 dilution; Alexis Biochemicals, San Diego, CA), GAPDH (1:500 dilution; clone 7B, LabFrontier, Seoul, Korea), ENO1 (1:1000 dilution; clone H300, Santa Cruz Biotechnology), 14-3-3 θ (1:1000 dilution; clone 3B9, Calbiochem, San Diego, CA), PSA (1:500 dilution; clone A0562, DakoCytomation), proteasome subunits 6, 2, 4, 5, 1, and 3 (1:2000 dilution; clone MCP231, Biomol International), PSMA1 (1:1000 dilution; clone MCP20, Biomol International), PSMA3 (1:1000 dilution; clone MCP72, Biomol

International), and proteasome subunit β1 (PSMB1; β6 according to the Baumeister *et al.*[132] nomenclature; Biomol International).

Isolation and analysis of exosomal RNA

Exosomal total RNA was isolated using the RNeasy Mini kit (Qiagen, Hilden, Germany) as described by Valadi *et al.*[128]. In short, pelleted exosomes were disrupted and homogenized in 350 µl of buffer RLT (Qiagen), and 1050 µl of 100% ethanol was added before samples were transferred to the RNeasy Mini spin column. Hereafter the procedure was followed as described by the manufacturer's protocol. Analysis of RNA expression was performed by RT-PCR. One microgram of exosomal RNA was reverse transcribed using Moloney murine leukaemia virus reverse transcriptase (Invitrogen) and an oligo- (dT)12 primer. Primer combinations used were as follows: PSA-4A (5-ACGTGTGTGCAAGTTCACC-3) and PSA-5B (5-TGTACAGGGAA-GGCCTTTCG- 3), TMPRSS2-E1 (5-AGCGCGGCAGGAAGCCTTA- 3) and ERG-R (5-GTAGGCACACTCAAACAACGACTGG-3), and GAPDH 462U17 (5-CATGT-TCGTCATGGGTG-3) and GAPDH 589L20 (5-ACTGTGGTCATGAGTCCTTC-3). PCR was performed for 27 cycles at an annealing temperature of 58 °C.

Results

Identification of 44 tumor-derived proteins in xenografted mice

The selection procedure followed to annotate identified proteins as tumour-derived in the circulation of human prostate cancer-xenografted mice is depicted in Figure 1. After serum collection from control mice (n=9) and PC346 (n=9) and PC339 (n=9) xenografted mice, samples were pooled and depleted of high abundance proteins, and proteins were separated by one-dimensional gel electrophoresis. Following tryptic digestion, peptides were subsequently analysed by LTQ-FT-ICR-MS/MS. After data analysis, 44 proteins were identified as tumour-derived (at greater than 95.0% probability and with two or more identified peptides) (Table 1). Of those, 22 were annotated as cytoplasmic proteins by the Gene Ontology database. The cytoplasmic proteins contained 12 of the subunits of the proteasome of which seven were identified based on the presence of human-specific peptides in the serum of xenograft-bearing mice.

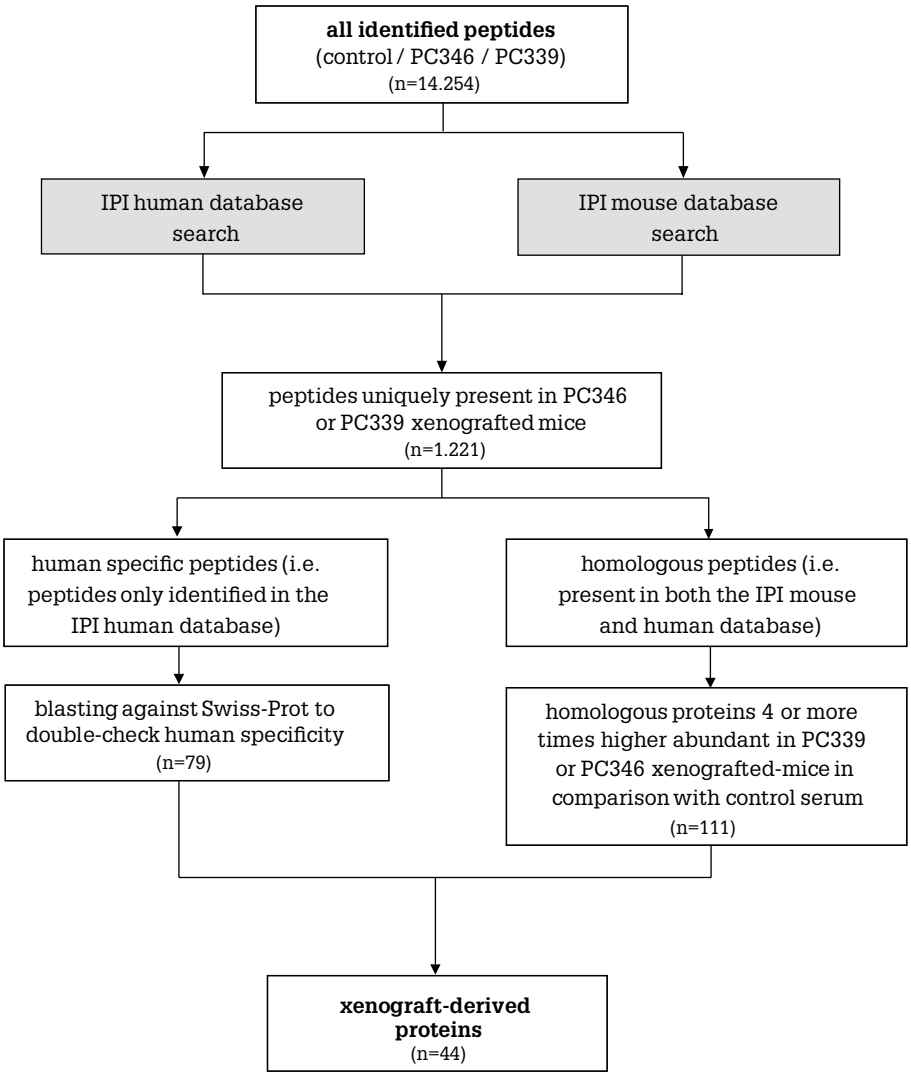

Figure 1. The selection procedure followed to annotate identified proteins as tumour-derived proteins.

Table 1. List of 44 proteins annotated as tumour-derived proteins in xenograft-bearing mice. Identified peptides were divided into a group of human specific peptides (identified only in the IPI human database) and a group of homologous peptides (present in both the IPI human and IPI mouse database). Homologous peptides were annotated as tumour-derived when 4 or more times higher abundant in the serum of PC339 or PC346 xenografted-mice in comparison with control serum. Annotations are derived from the Gene Ontology database. Proteins marked with an asterisk have earlier been identified by others in isolated exosomes from various origin.

Protein name	Gene symbol	IPI accession no.	Human/ homologue	No. of unique peptides
14-3-3 protein gamma*	YWHAG	IPI00230707	homologue	5
14-3-3 protein theta*	YWHAQ	IPI00018146	human	4
alpha-enolase*	ENO1	IPI00465248	human	4
apolipoprotein A-I precursor	APOA1	IPI00021841	human	3
cathepsin Z precursor	CTSZ	IPI00002745	homologue	2
chromosome 20 orf 114	C20orf114	IPI00291410	human	2
coactosin-like protein	COTL1	IPI00017704	homologue	2
coagulation factor V	F5	IPI00406603	homologue	6
cofilin, non-muscle isoform*	CFL1	IPI00012011	homologue	3
complement component C8 beta chain precursor	C8B	IPI00294395	homologue	2
cytochrome c	CYCS	IPI00465315	homologue	2
fructose-bisphosphate aldolase A*	ALDOA	IPI00465439	human/ homologue	11
glutathione peroxidase 3 precursor	GPX3	IPI00026199	human	5
glyceraldehyde-3-phosphate dehydrogenase*	GAPDH	IPI00219018	human	7
inter-alpha (globulin) inhibitor H3	ITIH3	IPI00028413	homologue	2
junction plakoglobin	JUP	IPI00554711	homologue	2

% Sequence coverage	Xenograft	Molecular Function	Biological Process	Location
20.65	PC339	protein kinase C binding	regulation of signal transduction	cytoplasm
15.92	PC339	protein kinase C inhibitor activity	regulation of progression through cell cycle	cytoplasm
26.67	PC339	phosphopyruvate hydratase activity	glycolysis	cytoplasm
16.24	PC339	lipid binding	cholesterol metabolism	secreted
5.61	PC346	cysteine-type peptidase activity	proteolysis	lysosome
4.34	PC346	lipid binding		
9.15	PC339	actin binding		
3.55	PC339	oxidoreductase activity	blood coagulation	secreted
21.69	PC339	protein binding	cytoskeleton organization and biogenesis	cytoplasm
5.66	PC339		immune response	secreted
17.14	PC346/339	heme binding	caspase activation via cytochrome c	mitochondrion matrix
30.77	PC346/339	fructose-bisphosphate aldolase activity	glycolysis	
23.01	PC339	gluthatione peroxidase activity	hydrogen peroxide catabolism	secreted
25.97	PC346/339	glyceraldehyde-3-phosphate dehydrogenase activity		cytoplasm
3.03	PC346	serine-type endopeptidase inhibitor activity	hyaluronan metabolism	secreted
2.81	PC339	cytoskeletal protein binding	cell adhesion	

Protein name	Gene symbol	IPI accession no.	Human/ homologue	No. of unique peptides
lactate dehydrogenase A*	LDHA	IPI00217966	human/ homologue	9
lactate dehydrogenase B*	LDHB	IPI00219217	human	10
lumican precursor	LUM	IPI00020986	homologue	2
lysozyme C precursor	LYZ	IPI00019038	human/ homologue	4
maltase-glucoamylase, intestinal*	MGAM	IPI00220143	human	3
myosin heavy chain, skeletal muscle, adult 2	MYH2	IPI00007856	homologue	3
myosin, light polypeptide 6, alkali, smooth muscle and non-muscle isoform 1	MYL6B	IPI00335168, IPI00413922	homologue	3
nucleoside diphosphate kinase A	NME1	IPI00012048	human/ homologue	6
nucleoside diphosphate kinase B	NME2	IPI00026260	homologue	6
peroxiredoxin-2*	PRDX2	IPI00000874, IPI00027350	human/ homologue	3
proteasome subunit alpha type 1	PSMA1	IPI00472442	human/ homologue	6
proteasome subunit alpha type 2	PSMA2	IPI00219622	homologue	5
proteasome subunit alpha type 4	PSMA4	IPI00299155	human	5
proteasome subunit alpha type 6	PSMA6	IPI00029623	homologue	9
proteasome subunit alpha type 7	PSMA7	IPI00024175	human	6
proteasome subunit beta type 1	PSMB1	IPI00025019	human/ homologue	7
proteasome subunit beta type 2	PSMB2	IPI00028006	homologue	2
proteasome subunit beta type 3*	PSMB3	IPI00028004	homologue	3
proteasome subunit beta type 4	PSMB4	IPI00556607	human/ homologue	5
proteasome subunit beta type 5	PSMB5	IPI00479306	human/ homologue	10
proteasome subunit beta type 6	PSMB6	IPI00000811	human	3
proteasome subunit beta type 8	PSMB8	IPI00000783	homologue	2
prothrombin precursor	F2	IPI00019568	homologue	2

% Sequence coverage	Xenograft	Molecular Function	Biological Process	Location
21.69	PC346/339	L-lactate dehydrogenase activity	anaerobic glycolysis	cytoplasm
29.94	PC346/339	L-lactate dehydrogenase activity	anaerobic glycolysis	cytoplasm
5.92	PC339	collagen binding	collagen fibril organization	secreted
23.65	PC346/339	lysozyme activity	inflammatory response	
1.18	PC346/339	protein binding	carbohydrate metabolism	cell membrane
4.94	PC346	actin binding		
14.42	PC339	structural component of muscle	muscle filament sliding	
46.71	PC339	nucleoside diphosphate kinase activity	negative regulation of cell proliferation	cytoplasm
42.76	PC339	nucleoside diphosphate kinase activity	negative regulation of cell proliferation	cytoplasm
13.13	PC339	thioredoxin peroxidase activity	anti-apoptosis	cytoplasm
24.91	PC346/339	peptidase activity	proteolysis	cytoplasm
22.64	PC346/339	peptidase activity	proteolysis	cytoplasm
17.30	PC339	peptidase activity	proteolysis	cytoplasm
37.80	PC346	peptidase activity	proteolysis	cytoplasm
21.37	PC339	peptidase activity	proteolysis	cytoplasm
34.02	PC346/339	peptidase activity	proteolysis	cytoplasm
12.44	PC346	peptidase activity	proteolysis	cytoplasm
18.05	PC346/339	peptidase activity	proteolysis	cytoplasm
17.80	PC346/339	peptidase activity	proteolysis	cytoplasm
38.78	PC346/339	peptidase activity	proteolysis	cytoplasm
12.97	PC346/339	peptidase activity	proteolysis	cytoplasm
5.88	PC346	protein binding	proteolysis	cytoplasm
2.25	PC346	thrombin activity	regulation of progression through cell cycle	secreted

Protein name	Gene symbol	IPI accession no.	Human/ homologue	No. of unique peptides
splice Isoform 1 of Complement factor B precursor	CFP	IPI00639937	homologue	2
thrombospondin-1 precursor*	THBS1	IPI00296099	homologue	7
transcobalamin-2	TCN2	IPI00136556	homologue	3
triosephosphate isomerase 1 variant *	TPI1	IPI00465028	human/ homologue	5
voltage-dependent anion channel 2	VDAC2	IPI00455531	homologue	2

% Sequence coverage	Xenograft	Molecular Function	Biological Process	Location
4.90	PC346/339	complement binding	complement activation	secreted
7.26	PC346/339	signal transducer activity	cell motility	secreted
6.09	PC339	cobalamin transporter activity	cobalamin transport	secreted
22.09	PC346/339	triose-phosphate isomerase activity	glycolysis	
7.77	PC346	voltage-gated anion channel porin activity	anion transport	mitochondrion outer membrane

Validation and characterization of proteasome subunits in xenograft sera

To specify and validate the presence of tumour-derived proteasome subunits in the circulation of the xenografted mice, two-dimensional SDS-PAGE analysis of xenograft-derived serum samples was performed. Figure 2 shows a comparison between serum from control mice (n=3) and serum from PC339 (n=3) and PC346 (n=3) xenografted mice.

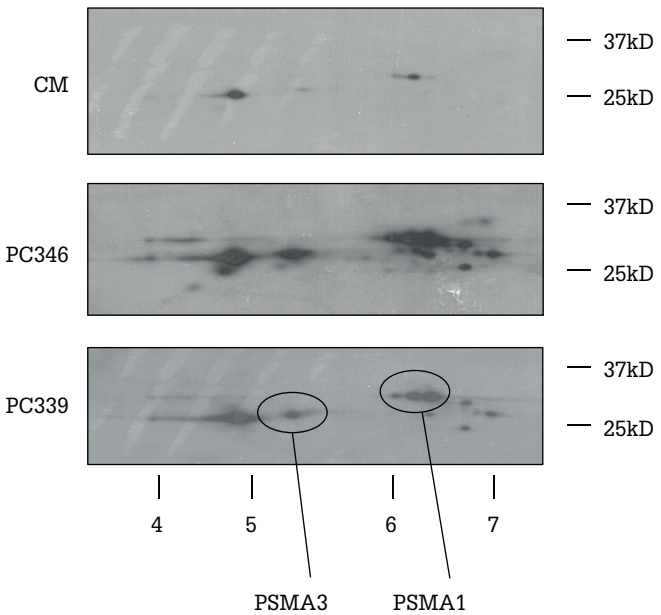

Figure 2. 2D PAGE Western blotting analysis of depleted serum from control mice (CM), PC346 xenograft-bearing mice (PC346), and PC339 xenograft-bearing mice (PC339). Spots were detected by using a monoclonal antibody to proteasome subunits 6, 2, 4, 5, 1, and 3. The presence of PSMA1 and PSMA3 proteasome subunits was confirmed with specific monoclonal antibodies. Strong signals are visible in the serum from both the PC346 and PC339 mice. Also faint signals were detected in the control serum because of crossreactivity with mouse proteasome subunits, which are present under normal conditions in the mouse serum.

Proteasome subunits were detected using a monoclonal antibody directed against subunits 6, 2, 4, 5, 1, and 3 of the proteasome. Strong signals were observed in the serum from both the PC346 and PC339 xenografted mice. As the proteasome antibody recognizes both mouse and human proteasome subunits, also faint signals were visible in the control serum that are known to be normally present in the mouse circulation. This is in line with the identification of mouse-specific proteasome peptides as detected by FT-ICRMS/ MS (data not shown). After stripping, blots were reprobed, and two of the spots could be specifically identified in the serum of PC339 and PC346 xenografted mice as the PSMA1 and PSMA3 subunits by using specific monoclonal antibodies directed against these proteins. The identified spots were consistent regarding molecular weight and pI with an earlier study performed by Claverol *et al*.[137].

Proteasome subunits are circulating as a complex in xenograft sera

To investigate whether the identified proteasome subunits were present as proteasome complexes in the serum of xenografted mice, native gel electrophoresis of control serum and serum from xenograft-bearing mice was performed. Figure 3a shows the presence of high molecular weight proteasome complexes in the xenograft sera. In both the 12.5% gel and 3.5% gel high molecular weight complexes are visible in the samples that were run under native conditions. After denaturation, the high molecular weight complexes disappeared, indicating disintegration into single proteasome subunits. The same effect is seen in the endogenous proteasome subunits of the control mouse. The presence of intact proteasome complexes was also investigated by sandwich ELISA.

Serum samples from control (n=3) and xenograft mice (n=6) were diluted 1:20 and analysed in triplicate under native conditions and after denaturation by heating at 96 °C for 5 min. Proteasome levels (mean ± S.D.) under native conditions in control, PC346, and PC339 serum were 10.5 ± 10.6, 32,759 ± 1720.0, and 20,339 ± 5062.2 ng/ml, respectively. After denaturation, proteasome concentrations (mean ± S.D.) decreased to 0.040 ± 0.03, 0.17 ± 0.16, and 0.28 ± 0.10 ng/ml in control, PC346, and PC339 serum, respectively (Figure 3b). Because the capture antibody of the sandwich ELISA is directed against the 1 subunit (PSMA1) of the proteasome whereas the detection antibody is directed against β subunits, this confirms the presence of proteasome complexes in the serum of the xenografted mice.

Electron microscopy of isolated exosomes

A large portion of the cytoplasmic tumour-derived proteins identified in the xenograft model has previously been identified as part of the human exosomal protein content. To explore the origin of the cytoplasmic proteins identified in the xenograft serum, exosomes were isolated from the PC346C cell line. To confirm that the structures isolated were indeed exosomes, they were examined by electron microscopy (Figure 4a). This showed a homogenous mixture of small bilayer membrane vesicles with an average diameter of 140 nm. ImmunoGold labelling of exosomes with an antibody to CD9 (Tetraspanin 29), an established marker for tumour cell-derived exosomes, showed positive exosome membrane staining (Figure 4a).

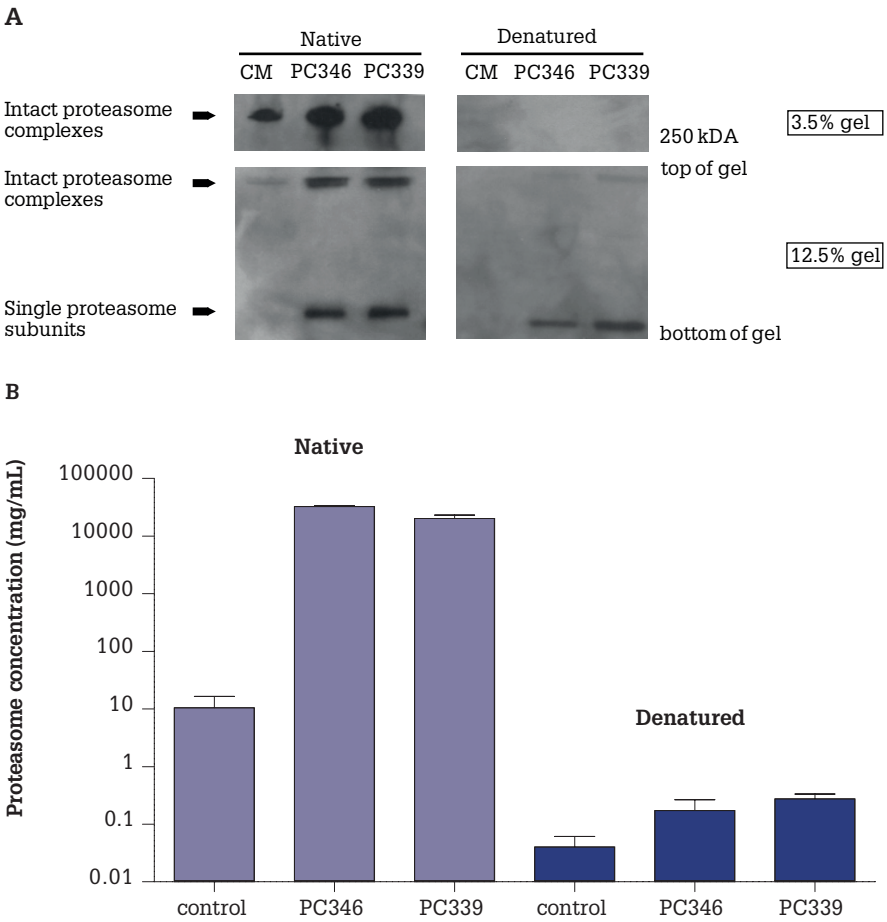

Figure 3. a, native 1D gel electrophoresis of serum from control mice (CM) and PC346 or PC339 xenograft-bearing mice showing the presence of intact proteasome complexes. High molecular weight complexes are visible under native conditions in both 12.5 and 3.5% gels. After denaturation, mostly single subunits are visible in the 12.5% gel, and the high molecular weight complexes in the 3.5% gel have disappeared. Bands were detected by a monoclonal antibody directed against the PSMA1 subunit of the proteasome. b, proteasome concentrations in xenograft-bearing mice and control mice serum samples under native (left) and denatured (right) conditions as measured by sandwich ELISA. After serum denaturation proteasome levels are strongly diminished, indicating the existence of proteasome complexes in xenograft and control serum samples. Error bars represent standard deviations.

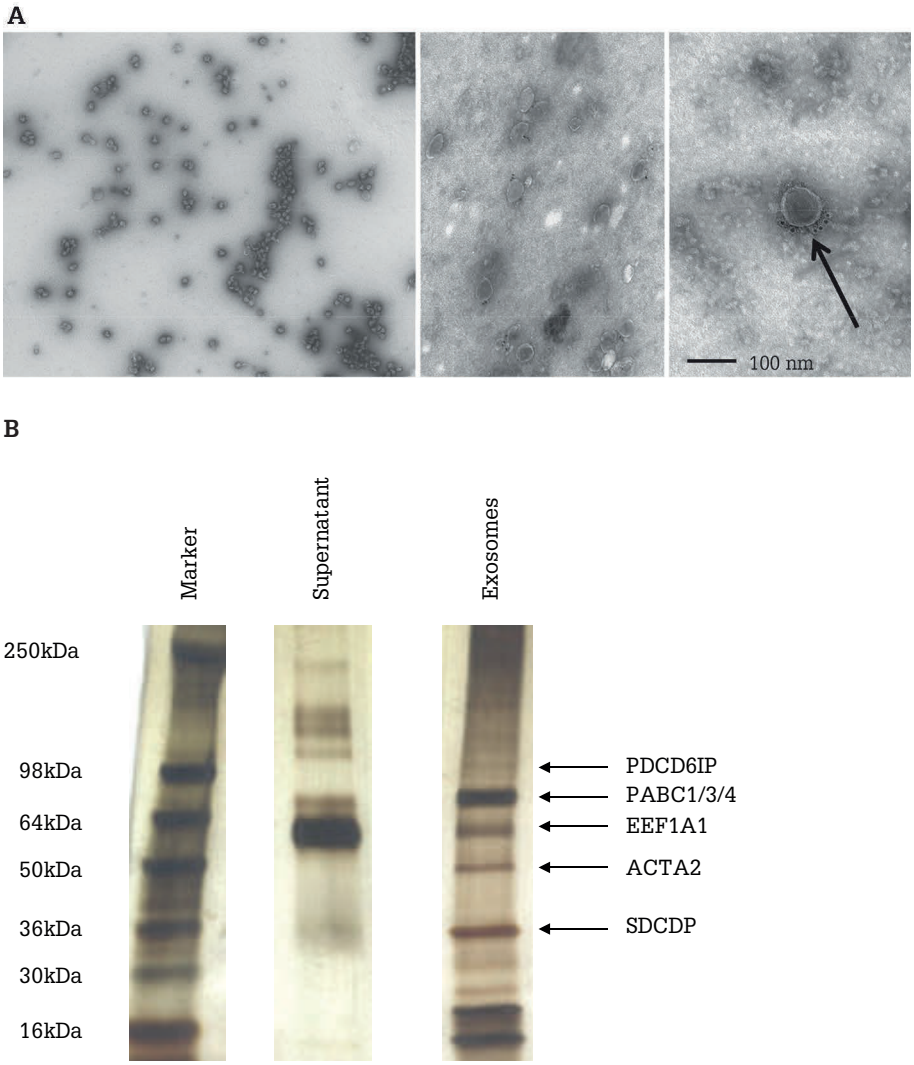

Figure 4. a, electron microscopy of exosomes isolated from the PC346C cell line. Left, electron micrograph of negatively stained exosomes showing a homogenous mixture of isolated vesicles; middle, ImmunoGold labelling of exosomes with the exosomal marker CD9 (Tetraspanin 29); right, increased magnification of the middle image shows positive CD9 membrane staining of exosomes (see arrow). b, separation of PC346C cell-derived exosomal proteins by 1D SDS-PAGE followed by silver staining. The indicated protein bands were excised and identified by LTQ-Orbitrap.

Proteomics analysis of exosomes

Supernatant and exosome fractions of the PC346C cell line were separated by 1D SDS-PAGE followed by silver staining (Figure 4b). The exosome fractions showed several distinct bands, which were absent in the supernatant fraction. Several bands were excised and subjected to LTQ-Orbitrap mass spectrometry (MS/MS) leading to the identification of five proteins: programmed cell death 6 protein (PDCD6IP; 10 unique peptides, 10.65% sequence coverage), poly(A)-binding protein 1 (PABC1; 12 unique peptides, 18.71% sequence coverage), eukaryotic translation elongation factor 1 α 1 (EEF1A1; three unique peptides, 6.28% sequence coverage), α-actin-2 (ACTA2; 13 unique peptides, 45.62% sequence coverage), and syndecan-binding protein (syntenin; four unique peptides, 14.77% sequence coverage). An in-depth proteomics analysis of the whole exosome fraction was performed by LTQ-FT-ICR-MS/MS. A total of 48 unique proteins were discovered in the exosome fraction of the PC346C cell line at a protein identification probability of 99% and 2 peptides per protein (Table 2). At a protein probability of 99% and 1 peptide per protein 126 proteins were identified. Among those proteins identified with two or more peptides per protein were two of the proteins that had earlier been identified in the serum of xenograft-bearing mice (GAPDH and lactate dehydrogenase B)[124]. Also the presence of the exosomal marker CD9 and the prostate-specific protein folate hydrolase 1 (FOLH1; prostate-specific membrane antigen) were confirmed. ENO1 and fructose-bisphosphate aldolase A, also previously identified in the serum of xenograft-bearing mice, were positively identified at a probability of one peptide per protein (data not shown). All proteins identified in the specific exosome bands but ACTA2 (Figure 4b) were also recovered in the in-depth proteomics analysis.

Table 2. List of 48 proteins identified by two or more peptides in the exosome fraction of the PC346C cell line. Annotations are derived from the Gene Ontology database.

Protein name	Gene symbol	Protein accession numbers	No. of unique peptides	% Sequence coverage
Actin, gamma 1	ACTG1	IPI00021440	15	40.30
ADAM metallopeptidase domain 10	ADAM10	IPI00013897	3	3.88
ADAM metallopeptidase domain 15	ADAM15	IPI00013302	4	6.39
Annexin A2	ANXA2	IPI00418169	7	24.90
Annexin A6	ANXA6	IPI00002459	3	7.20
ATPase, Na+/K+ transporting, alpha 1 polypeptide	ATP1A1	IPI00006482	5	5.96
Brain abundant, membrane attached signal protein 1	BASP1	IPI00299024	4	40.10
Chromosome 1 open reading frame 58	C1orf58	IPI00065500	2	10.70
Capping protein (actin filament) muscle Z-line, alpha 1	CAPZA1	IPI00005969	2	7.69
CD151 molecule (Tetraspanin 24)	CD151	IPI00298851	2	5.93
CD2-associated protein	CD2AP	IPI00412771	6	11.60
CD9 molecule	CD9	IPI00215997	3	15.40
Chromatin modifying protein 4B	CHMP4B	IPI00025974	2	8.93
Clathrin, heavy chain 1	CLTC	IPI00024067	10	6.81
Eukaryotic translation elongation factor 1 alpha 1	EEF1A1	IPI00396485	14	37.20
EH domain-containing protein 1	EHD1	IPI00017184	2	5.06
F11 receptor	F11R	IPI00001754	5	16.40
Family with sequence similarity 125, member A	FAM125A	IPI00744702	3	16.10
Formin binding protein 1-like	FNBP1L	IPI00015580	2	3.47
Folate hydrolase (prostate-specific membrane antigen) 1	FOLH1	IPI00028514	7	12.30
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	IPI00219018	3	14.00
Histone cluster 1, H1c	HIST1H1C	IPI00217465	2	10.30

Molecular Function	Biological Process	Location
structural constituent of cytoskeleton	cell motility	cytoplasm
protein homodimerization activity	protein amino acid phosphorylation	cell membrane
proteolysis and peptidolysis	cell adhesion	cell membrane
phospholipase inhibitor activity	skeletal development	secreted protein
calcium ion binding		
sodium:potassium-exchanging ATPase activity	sodium ion transport	cell membrane
		cell membrane
actin binding	cell motility	
protein binding	cell adhesion	cell membrane
structural constituent of cytoskeleton	cell migration	cytoplasm
protein binding	cell motility	cell membrane
		cytoplasm
signal transducer activity	receptor-mediated endocytosis	cell membrane
translation elongation factor activity	translation elongation	cytoplasm
ATP binding		cell membrane
	cell motility	cell membrane
		cytoplasm
		cytoplasm
dipeptidase activity	proteolysis	cell membrane
glyceraldehyde-3-phosphate dehydrogenase activity	glycolysis	cytoplasm
DNA binding	nucleosome assembly	nucleus

Protein name	Gene symbol	Protein accession numbers	No. of unique peptides	% Sequence coverage
Histone cluster 1, H2ab	HIST1H2AB	IPI00026272	2	21.50
Heat shock protein 90kDa alpha (cytosolic), class B member 1	HSP90AB1	IPI00334775	4	6.49
Heat shock 70kDa protein 1B	HSPA1B	IPI00807640	6	16.50
Heat shock 70kDa protein 8	HSPA8	IPI00003865	13	25.50
Immunoglobulin superfamily, member 8	IGSF8	IPI00056478	6	12.60
Integrin beta-1	ITGB1	IPI00217563	3	3.89
Lactate dehydrogenase B	LDHB	IPI00219217	2	7.78
Milk fat globule-EGF factor 8 prot	MFGE8	IPI00002236	2	4.91
Poly(A) binding protein, cytoplasmic 1	PABPC1	IPI00008524	16	25.90
Poly(A) binding protein, cytoplasmic 4	PABPC4	IPI00555747	5	18.40
Protein kinase C and casein kinase substrate in neurons 2	PACSIN2	IPI00027009	4	8.85
Poly(rC)-binding protein 2	PCBP2	IPI00012066	2	7.46
Programmed cell death 6 interacting protein	PDCD6IP	IPI00246058	21	22.40
Prostaglandin F2 receptor negative regulator	PTGFRN	IPI00022048	7	9.33
Ribosomal protein S27a	RPS27A	IPI00179330	5	31.40
Syndecan binding protein (syntenin)	SDCBP	IPI00299086	3	15.10
Serine incorporator 5	SERINC5	IPI00328883	2	4.97
SH3-domain GRB2-like 1	SH3GL1	IPI00019169	8	23.60
Solute carrier family 3, member 2	SLC3A2	IPI00027493	2	5.48
Sphingomyelin phosphodiesterase, acid-like 3B	SMPDL3B	IPI00550115	2	4.73
Sorting nexin-9	SNX9	IPI00001883	2	5.38
Tumor-associated calcium signal transducer 1	TACSTD1	IPI00296215	5	21.30
Tumor susceptibility gene 101 protein	TSG101	IPI00018434	7	21.30
Tubulin, beta	TUBB	IPI00011654	3	7.43
Vacuolar protein sorting 37 homolog B	VPS37B	IPI00002926	3	10.20
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	YWHAZ	IPI00021263	5	22.90

Molecular Function	Biological Process	Location
DNA binding	nucleosome assembly	nucleus
unfolded protein binding	response to unfolded protein	cytoplasm
unfolded protein binding	anti-apoptosis	cytoplasm
ATPase activity	protein folding	cytoplasm
protein binding	cell motility	cell membrane
protein heterodimerization activity	cell migration	cell membrane
L-lactate dehydrogenase activity	anaerobic glycolysis	cytoplasm
	cell adhesion	cell membrane
translation activator activity	mRNA stabilization	cytoplasm
protein / RNA binding	RNA processing	cytoplasm
transporter activity	intracellular protein transport	cytoplasm
protein / RNA binding	mRNA metabolic process	cytoplasm
signal transducer activity	apoptosis	cytoplasm
protein binding	negative regulation of protein biosynthetic process	cell membrane
structural constituent of ribosome	translation	cytoplasm
protein heterodimerization activity	cell migration	cell membrane
		cell membrane
protein binding	signal transduction	cell membrane
catalytic activity	amino acid transport	cell membrane
hydrolase activity	carbohydrate metabolism	secreted protein
protein binding	protein localization	
		cell membrane
transcription corepressor activity	regulation of cell growth	cytoplasm
structural constituent of cytoskeleton	spindle assembly	
transcription factor binding	signal transduction	cytoplasm

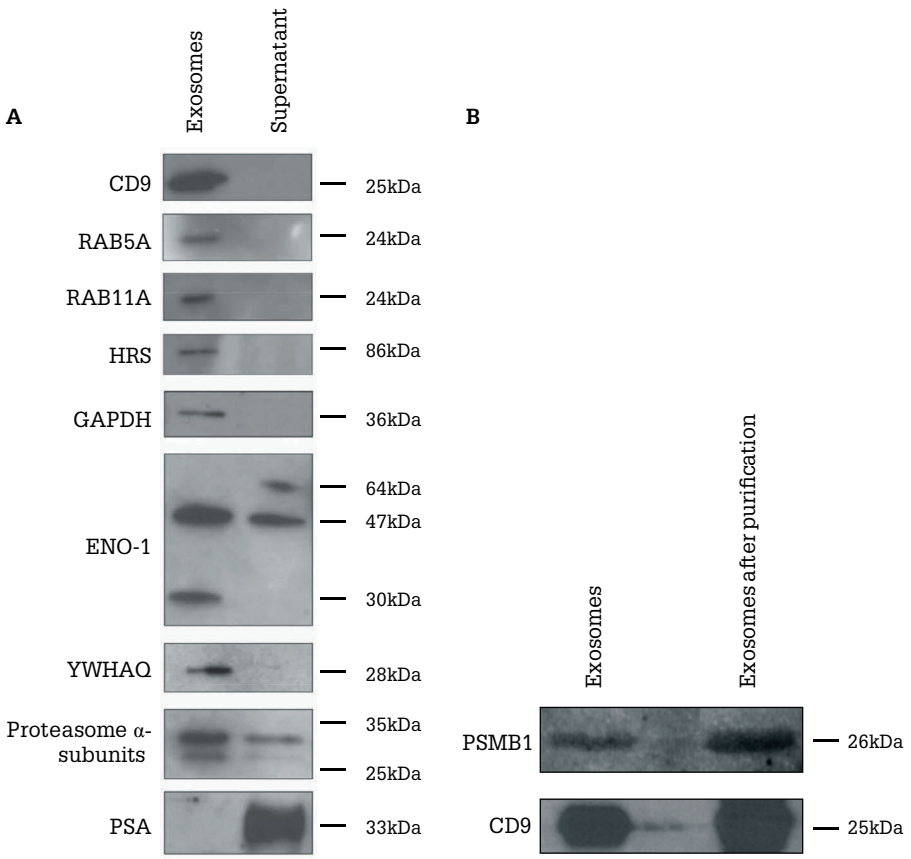

Figure 5. a, 1D PAGE and Western blotting analysis comparing the exosome and supernatant fractions of the PC346C cell line for CD9, the members of the RAS oncogene family RAB5A and RAB11A, HRS, GAPDH, ENO1, YWHAQ (14-3-3 protein θ), proteasome subunits, and PSA. CD9, RAB5A, RAB11A, HRS, GAPDH, and YWHAQ were uniquely identified in the isolated exosomes, whereas PSA could only be detected in the supernatant of the PC346 cell line. b, 1D PAGE and Western blotting analysis of the exosome fraction after purification with magnetic beads. This figure shows that the CD9 and proteasome β1 (PSMB1) signals are visible in the exosome fraction both before and after purification with magnetic beads, indicating that proteasome subunits are present inside exosomes or exosomal membranes.

Analysis of exosomes by one-dimensional SDS-PAGE analysis

One-dimensional SDS-PAGE and Western blotting were performed to verify the presence in exosomes of several proteins previously identified by LTQ-FT-ICR-MS/MS in the xenograft model. CD9, RAB5A, RAB11A, HRS, GAPDH, and 14-3-3 protein θ (YWHAQ) were uniquely present in the isolated exosome fraction and could not be detected in the PC346C cell line supernatant, whereas α-enolase (ENO1) and subunits of the proteasome were present in both fractions. PSA was uniquely present in the supernatant fraction and could not be detected in the isolated exosomes (Figure 5a). To certify that the proteasome subunits present in the exosome fraction were not the result of simultaneous pelleting of exosomes and proteasome complexes during ultracentrifugation, exosomes were further purified by magnetic beads coated with a CD9 antibody. After exosomal purification, bead-exosome complexes were loaded onto one-dimensional SDS-polyacrylamide gels. Blots were incubated with a monoclonal antibody to CD9 (Chemicon International) or a polyclonal antibody against the PSMB1 subunit of the proteasome. Figure 5b shows that both the CD9 and PSMB1 subunit signals are visible in the exosome fraction as well as in the immunobead-purified exosome fraction.

Analysis of exosomal RNA

As the PCa-specific TMPRSS2:ERG gene fusion is expressed in the majority of PCa patients, we analysed exosomes for the presence of the gene fusion product[70,138]. RNA was isolated from PC346C and VCaP cells and analysed by RT-PCR. Both cell lines express PSA, whereas the TMPRSS2:ERG gene fusion is only present in VCaP and not in PC346 cells[138]. KLK3 (PSA) and GAPDH RNAs were present in both VCaP and PC346C exosomes as well as in the total RNA fraction from both cell lines. The gene fusion product TMPRSS2:ERG was only detected in VCaP exosomes and in the VCaP cell line and was not detected in PC346C-derived exosomes and the PC346C cell line (Figure 6).

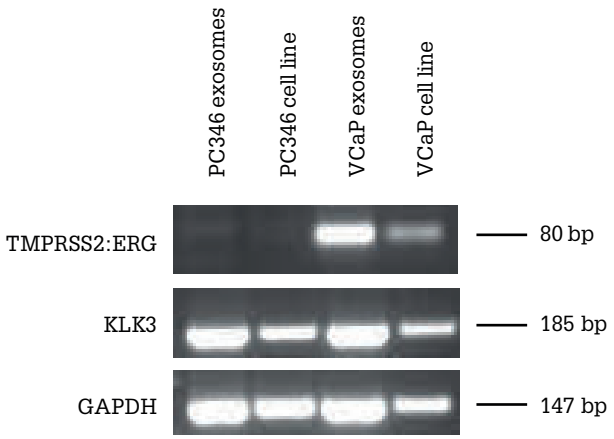

Figure 6. RT-PCR analysis of VCaP and PC346C cell lines and exosomes. The TMPRSS2:ERG fusion gene is exclusively expressed in both the VCaP cell line and exosomes, whereas both cell lines and exosomes express KLK3 (PSA) and GAPDH. H2O and RT controls were negative (data not shown).

Discussion

The present study shows the identification of 44 tumour-derived proteins by mass spectrometry in xenograft models for PCa. Virtually all subunits of the proteasome were among the proteins identified, a finding that was verified by two-dimensional gel electrophoresis of xenograft-bearing and control mouse sera. Several of these proteasome subunits are part of the normal human plasma proteome as was shown by the Human Proteome Organisation Plasma Proteome Project[125]. Increased proteasome levels have been related to haematological malignancies, especially multiple myeloma[139], but also to solid tumours, such as melanoma and colon carcinoma[140]. Recently Byrne *et al.*[141] identified the proteasome β6 subunit in a proteomics analysis of serum from patients with PCa. Abnormal gene expression of proteasome subunits has been reported in several cancer types[139,142]. High plasma proteasome levels reflect the dysregulation of protein synthesis and degradation in cancer cells in contrast to normal cells in which the proteasome complex plays a crucial role in controlling essential cellular functions such as transcription, stress response, cell cycle regulation, cellular differentiation, and DNA repair[143]. This is also illustrated by the fact that in malignancies proteasome inhibitors induce apoptosis, have in vivo antitumour efficacy, and sensitize malignant cells for conventional therapies[143]. The secretion mechanism of circulating proteasomes in

cancer patients and healthy donors is still unknown. Elevated proteasome concentrations in culture media of human leukemic cell lines have been reported, suggesting a proteasome secretion mechanism by tumour cells[144]. In this study, we have shown, using native gel electrophoresis and sandwich ELISA of sera from xenografted and control male mice, that at least part of the circulating proteasome subunits are present as proteasome complexes. This is in line with the observation that circulating proteasomes are intact and enzymatically active in plasma from healthy donors and patients with autoimmune disease or leukaemia[145,146]. About half of the tumour-derived proteins circulating in the xenograft-bearing mice, including proteasome subunits, are not secreted proteins but are annotated as being cytoplasmic. One possible explanation for their presence could be the occurrence of necrosis or apoptosis in the xenografts, a well known characteristic of most cancers. Although we did not find evidence for necrosis in the xenografts used for this study, protein secretion via cell death cannot be excluded. Microarray expression data of the identified cytoplasmic proteins showed that these indeed corresponded to genes that are highly expressed in PCa. However, none of the proteins of the 100 most highly expressed genes in PCa (such as ribosomal and cytoskeletal genes) have been identified in the circulation of the xenograft-bearing mice (data not shown). One would expect to detect the proteins of these highly expressed genes if these are the result of tumour apoptosis. Although this does not rule out the contribution of apoptosis or necrosis, this points toward certain specific processes responsible for the secretion of cytoplasmic proteins. We hypothesized that one such specific process could be the secretion of proteins via exosomes. A literature search revealed that 13 of the 44 (30%) identified proteins in the xenograft model had earlier been identified in exosomes among which was also the β3 subunit of the proteasome (Table 1). First, we showed that the human PCa cell line PC346C is indeed capable of the secretion of exosomes (Figure 4a). Second, LTQ-FT-ICR-MS/MS and Western blotting analyses of isolated exosomes showed the presence of mainly cytoplasmic proteins, including GAPDH, fructose-bisphosphate aldolase A, ENO1, lactate dehydrogenase B, 14-3-3 protein θ, and proteasome subunits (see Table 2 and Figure 5a). Also the exosomal marker CD9 and several proteins up-regulated in PCa, among which was FOLH1, were identified. In addition RAB5A, RAB11A, and HRS, proteins involved in vesicular and endosomal trafficking, were present in the exosome fraction as shown by Western blotting (Figure 5a). The detection of HRS by Western blotting confirmed the identification of HRS by LTQ-FT-ICR-MS/MS at a setting of one peptide per protein (data not shown). To argue against the fact that the proteasome signal in the exosome fraction was the result of simultaneous pelleting of exosomes and proteasome complexes, exosomes were further purified by immunoaffinity precipitation utilizing anti-CD9 antibody-coated magnetic beads.

This strengthened our finding that proteasomes are present inside exosomes and/or tightly associated with exosomal membranes or external macromolecules. Our observation is in agreement with Almeida *et al*.[147] and Dong *et al*.[148] who showed the presence of proteasomes in late endosomes from which exosomes are formed by invagination and budding. To our knowledge, the present study is the first to describe a specific clearance mechanism for proteasome subunits in cancer cells, providing a possible explanation for the increased proteasome serum levels that have been observed in several types of cancer patients. An important observation is that PSA was not detectable in the exosome pellets but was abundant in the supernatant. This means that we experimentally separated protein secretion via two different secretion pathways: (i) the typical secretion of signal peptide-containing proteins via the rough endoplasmic reticulum and Golgi apparatus secretory pathway and (ii) the multivesicular body-exosome secretion route. This is in line with our observation that of a total of 48 proteins identified in the exosome fraction the majority are annotated as cytoplasmic whereas only two proteins are annotated as secreted according to the Gene Ontology database. In contrast, 10 of 44 tumour-derived proteins identified in the serum of xenograft-bearing mice are annotated as secreted proteins (Tables 1 and 2). Thus, it seems that several of the cytoplasmic proteins, including proteasome subunits, present in xenograft sera are at least partly secreted via the exosome pathway in contrast with proteins such as PSA that are typically secreted via the Golgi consecutive secretory pathway. The putative in vivo analogues of exosomes could be prostasomes, secreted vesicles in human seminal fluid, secreted by epithelial prostate cells. Proteomics analysis of human prostasomes revealed 139 proteins, showing a small overlap (7 of 139) with the proteins identified in exosomes in the present study. Among the proteins identified in prostasomes were several glycolysis-related enzymes, heat-shock proteins, and proteins of the annexin family[149]. In contrast, no proteasome subunits or members of the tetraspanin family were identified in prostasomes isolated from seminal fluid. It was recently reported by Valadi *et al*.[128] that exosomes not only contain proteins but also mRNAs, suggesting a potential novel mechanism of genetic exchange between cells. By RNase and trypsin treatment of the isolated exosomes it was confirmed that the mRNA was indeed confined within exosomes and not on external structures or macromolecules. This prompted us to isolate exosomal RNA from the PC346C and VCaP cell lines. Of these cell lines it has been shown that the VCaP cell line expresses the TMPRSS2:ERG fusion gene, whereas PC346C does not[70,138]. RT-PCR analysis correctly showed that the PCa-specific TMPRSS2:ERG fusion gene is present in exosomal RNA from the VCaP cell line whereas it is absent in exosomal RNA from the PC346C cell line. As it has been reported that exosomes exist in human serum, prostate-specific proteins present in

the membrane of exosomes, such as FOLH1, could be used to isolate prostate-specific exosomes to discover and validate new markers for PCa[150]. For example, this could lead to the development of a serum test for RNA transcribed from the TMPRSS2:ERG gene fusion. The specific role of exosomes in cancer is still not fully understood, and involvement in processes such as cell-cell communication and antigen representation has been suggested[126-128]. The content of exosomes may represent a fingerprint of the cytoplasm of the cancer cell and may establish a unique environment that allows for the occurrence of specific processes. For example, Stoeck *et al.*[151] showed that exosomes may be a platform for ectodomain shedding of transmembrane proteins. In the present study, the presence of a distinct 30-kDa ENO1 band specifically present in the exosome fraction may point toward the occurrence of unique proteolytic activity inside exosomes (Figure 5a). It has recently been suggested that exosomes secreted from cancer cells support immune escape as well as tumour growth[127]. Also exosomes have been reported to induce angiogenesis and to transfer metastatic activity from highly to poorly metastatic tumour cells[152]. Of the proteins identified in the exosomes in the present study, CD151 and the metalloproteinases ADAM10 and ADAM15 have been linked to tumour invasiveness and prognosis[153-155]. The proteasomes identified in exosomes may harbour a similar function. In conclusion, the present study shows that, although their function is unclear, exosomes offer unique possibilities for PCa biomarker discovery as they give insight information about the interior of the cancer cell both on the protein and RNA levels. Future studies will focus on the validation of several identified exosomal proteins as well as on the detection of exosomal RNA, including the TMPRSS2:ERG and other gene fusion transcripts in exosomes isolated from patient populations, to establish new biomarkers for PCa diagnosis and prognosis.

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CHAPTER 3

PROFILING OF ANTIBODY PRODUCTION AGAINST XENOGRAFT-RELEASED PROTEINS BY PROTEIN MICROARRAYS DISCOVERS PROSTATE CANCER MARKERS

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Abstract

This study describes a novel xenograft-based biomarker discovery platform and proves its usefulness in the discovery of serum markers for prostate cancer. By immunizing immuno-competent mice with serum from nude mice bearing prostate cancer xenografts, an antibody response against xenograft-derived antigens was elicited. By probing protein microarrays with serum from immunized mice, several prostate cancer-derived antigens were identified, of which a subset was successfully retrieved in serum from mice bearing prostate cancer xenografts and prevalidated in human serum samples of prostate cancer patients. Among the discovered and validated proteins were the members of the TAM receptor family (TYRO3, AXL, and MERTK), ACY1, and PSMA1. In conclusion, this novel method allows for the identification of low abundant cancer-derived serum proteins, circumventing dynamic range and host-response issues in standard patient cohort proteomics comparisons.

Introduction

For several decades, prostate specific antigen (PSA) is the "gold standard" biomarker for the detection of prostate cancer (PCa)[13]. However, additional biomarkers are urgently needed, as PSA lacks diagnostic specificity and prognostic value, causing an enormous increase in unnecessary biopsies and overtreatment of low-risk PCa patients[26]. A possible approach to discover new serum-shed markers for PCa is by screening large series of human serum samples for tumour-associated proteins. However, this approach is hampered by the large variability and complexity of protein content, with a vast dynamic range in protein concentrations (>10^{10}) and dominance of a small number of high abundant proteins that constitute 99% of the entire protein content, masking the detection of other proteins[107]. Due to these high abundant proteins and unequal protein concentrations, current technologies, capable of analyzing protein concentrations spanning four orders of magnitude, can only detect proteins ranging over two orders of magnitude, missing the lower abundant proteins (such as PSA) which are likely to constitute the next generation of biomarkers. In general, studies exploiting this approach so far have only reported changes in relatively abundant and/or acute phase proteins[156-159].

To evade these dilemmas, xenograft model systems have been exploited to discover new biomarkers, by comparing serum of tumour-bearing with serum of non-tumour-bearing mice[124,160,161]. Although more specific and with less variability, this approach is still hampered by serum protein complexity and with standard liquid chromatography mass spectroscopy (LC-MS) technology will only result in detection of high abundant proteins.

In analogy to previous efforts to detect tumour-associated markers, we also utilized the highly sensitive and specific mouse immune system by developing a xenograft-based biomarker discovery platform, in which immuno-competent mice are immunized with serum from PCa xenograft-bearing nude mice (Figure 1)[162]. As the donor and recipient mice are congenic, human PCa-derived proteins will generate immunoglobulin biomarkers. After an initial verification of the immune response, a protein microarray based approach was utilized to profile the antibody production and identify PCa xenograft-derived antigens. Subsequently, several identified candidate biomarkers were pre-validated on serum samples of men with and without PCa.

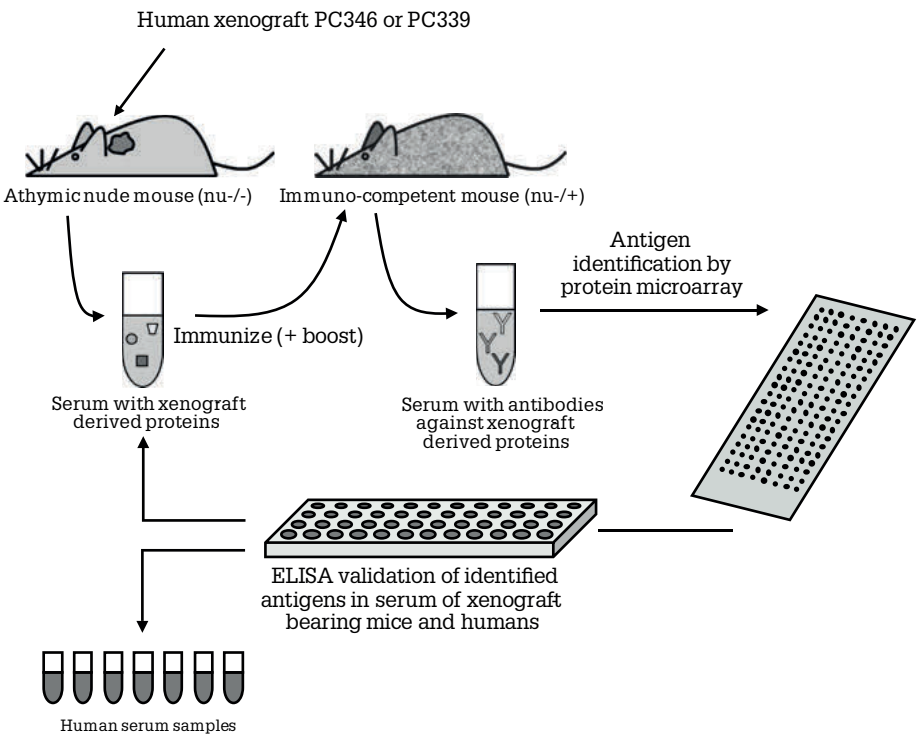


Figure 1. Study outline.
Antibodies against xenograft-derived proteins were generated after immunization of immuno-competent mice with serum from nude mice harbouring human PCa xenografts. A large scale identification of generated antibodies was performed by utilizing protein microarrays. Identified xenograft-derived antigens were validated in serum of xenograft-bearing mice and in human serum samples of men with and without PCa.

Methods

Immunization protocol

An overview of the animal experiments is given in Figure 1. Human PCa xenografts were grown in immune-incompetent athymic male nude (nu/nu) BALB/c mice (n=3 for each xenograft, Taconic, Ry, Denmark)[123,124]. We utilized the human PCa cell lines PC346 (androgen-sensitive) and PC339 (androgen-independent). Specific characteristics of both cell lines have been described previously[129]. Prior to tumour inoculation, control serum was collected by retro-orbital punction. Tumour-bearing mice were sacrificed when tumours reached a volume of 500-700 mm^3 and blood was collected (annotated as PC346 mouse serum and PC339 mouse serum, respectively). A portion of the collected serum was depleted from high-abundant serum proteins. A portion of the collected serum was depleted from high-abundant serum proteins utilizing Multi Affinity Removal Spin Cartridges (Agilent Technologies, Wilmington, DE, USA) according to manufacturer's instructions. Depleted serum samples were concentrated on 5 kDa cut off ultracentrifugation columns (Agilent Technologies, Wilmington, DE, USA). All samples were stored at -80°C.

Ten immuno-competent male nude (nu/+) BALB/c mice were immunized with pools (n=3) of either depleted control serum (two mice, annotated as control boost), depleted PC346 serum (three mice, annotated as PC346 boost depleted), depleted PC339 serum (three mice, annotated as PC339 boost depleted), full PC346 serum (one mouse, annotated as PC346 boost full) or full PC339 serum (one mouse, annotated as PC339 boost full). Prior to immunization, preimmune serum was collected. For immunization, mice were subcutaneously injected with 100 µl of a mixture of 10 µg serum and 50 µl complete Freund's adjuvant. After 3 weeks a boost-immunization was performed with a mixture of 10 µg serum and 50 µl incomplete Freund's adjuvant. After 6 weeks a second boost-immunization with full serum derived from PC346 or PC339 bearing mice was performed in two mice previously immunized with either depleted PC346 or PC339 serum (annotated as PC346 depleted-full or PC339 depleted-full, respectively). Four weeks after the last boost-immunization, mice were sacrificed and serum was collected. The protocol was approved by the Animal Experiments Committee under the national Experiments on Animals Act (DEC EUR 310; OZP102-04-03 and EUR 593; OZP102-05-12) and adhered to the rules laid down in this national law that serves the implementation of "Guidelines on the protection of experimental animals" by the council of Europe under Directive 86/609/EC.

Mouse serum concentration

For several experiments, 15 times concentrated mouse serum was utilized. This was obtained by concentration of 4,5 mL of either pooled control mouse serum or serum from mice inoculated with a PC346 or PC339 xenografts. Concentration was performed on 5 kDa cut off ultracentrifugation columns (Agilent Technologies). All samples were stored at -80°C.

One-dimensional SDS-PAGE analysis and western blotting

For one-dimensional electrophoresis 10 µg of PC346 and LNCaP cell line protein extracts was mixed with Laemmli sample buffer (1:1 ratio) and loaded onto a 10% SDS-PAGE gel[163]. Gels were transferred onto a Protran nitrocellulose membrane for western blotting. Blots were developed with 1:250 diluted serum samples derived from either control mice or mice immunized with serum from mice bearing PC346 or PC339 xenografts. Blots were exposed for equal amounts of time.

Measurement of serum PSA concentrations and detection of antibodies against PSA

From each mouse utilized in the immunization protocol (normal nude mice, and mice grafted with a PC339 or PC346 xenograft) 10 µl of serum was taken to measure serum PSA concentrations. PSA concentrations were determined by routine clinical chemistry applying the Access Hybritech Total PSA assay (Beckman-Coulter, Inc., Fullerton, CA) which has a lower detection limit of 0.10 ng/ml.

To detect antibodies directed against PSA in mice immunized with serum from mice inoculated with a PC346 or PC339 xenograft, 96-well plates were coated with 300 ng of purified PSA (Sigma-Aldrich, Milan, Italy) diluted in 0.1 M carbonate-bicarbonate buffer (pH 9.5) and incubated overnight at 4°C. After washing with PBS-Tween (0.05%), plates were blocked for 1 hour by addition of a 1% BSA/PBS-Tween (0.05%) solution and incubated at 37 °C. Thereafter, serum samples diluted in PBS-Tween were added and incubated for 1 hour at 37 °C. As a reference standard, a dilution of a polyclonal rabbit antibody directed against PSA (DakoCytomation, Glostrup, Denmark) was utilized. Then, a 1:5,000 solution in 0.5% BSA/PBS-Tween of HRP conjugated goat anti-rabbit IgG or goat-anti mouse antibody (DakoCytomation) was added and samples were incubated for 1 hr in the dark. To reveal HRP activity, 50 mM phosphate / 25 mM citrate buffer, pH 5.0 containing 4 mM o-phenylenediamine and 0.0015% H_2O_2 was added to the wells. After 15 min, the reaction was stopped with 2.5 M sulphuric acid. Absorbance values were measured at 492 nm and PSA antibody levels determined as compared to the dilution series of the commercial PSA polyclonal rabbit antibody reference. All samples were measured in duplo.

Detection of antibodies against human PCa xenograft-derived antigens

For immune response profiling of immunized mice ProtoArrays were utilized (Human Protein Microarray version 4.0, Invitrogen Life Technologies, Carlsbad, CA). The 4.0 version contains approximately 8,000 unique human proteins immobilized on a hydrophobic surface. Proteins are expressed as GST fusion proteins, purified and double spotted within distinct subarrays with a number of assay-specific positive and negative controls. ProtoArrays were developed according to the protocol of the manufacturer. In short, after blocking, arrays were incubated with serum 1:500 diluted in PBS with 1% BSA and 0.1% Tween. ProtoArrays were developed using two pools both consisting of two serum samples obtained prior to immunization (annotated preimmune (1) and (2)), a pool of two serum samples obtained from mice immunized with depleted serum from mice without a xenograft (control boost), two serum samples obtained from mice immunized with depleted serum from mice carrying either a PC346 or PC339 xenograft (PC346 depleted and PC339 depleted, respectively), two serum samples obtained from mice immunized with full serum from mice carrying either a PC346 or PC339 xenograft (PC346 full and PC339 full, respectively), and two serum samples obtained from mice immunized with depleted and full serum from mice carrying either a PC346 or PC339 xenograft (annotated as PC346 depleted-full and PC339 depleted-full, respectively). After 90 minutes at 4°C, arrays were washed and incubated with Alexa Fluor 647 conjugated rabbit anti-mouse IgG (H+L) (Invitrogen) 1:2,000 diluted in PBS-Tween with 1% BSA for 90 minutes at 4°C. As a control, one ProtoArray was developed without serum incubation utilizing only the Alexa Fluor 647 conjugated rabbit anti-mouse IgG 2nd antibody. After washing, arrays were dried and immediately scanned in a ScanArray Express HT (Perkin Elmer, The Netherlands). Spot intensities were quantified using Prospector Imager (version 4.0, Invitrogen) and ProtoArray Prospector (version 4.0.0, Invitrogen). Spot signals were normalized per mean array intensity. Spots were sorted based on their preferential expression in mice immunized with serum from xenograft-bearing animals vs. their expression in preimmune serum and serum from mice immunized with serum from non-xenograft bearing mice. Spots with a normalized expression value of >6 in either the preimmune (1), preimmune (2) or control boost samples were excluded as well as spots with a normalized expression <2 in mice immunized with serum from xenograft-bearing animals. The normalized expression value of one of the spots developed with serum from xenograft-bearing animals was set to exceed a value of three. The ProtoArray data discussed in this publication are MIAME (Minimum Information About a Microarray Experiment) compliant, have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE16220 (http://www.ncbi.nlm.nih.gov/geo/).

Patient samples

Serum samples of men without PCa were obtained from the biorepository of the Rotterdam arm of the ERSPC[164]. The medical ethical committee of the Erasmus University Medical Centre approved the ERSPC study (MEC number 138.741/1994/152). All participants provided written informed consent. To exclude the presence of PCa in these men (n=40), PSA serum concentrations had to be below 0.4 ng/ml and no tumour had to be detected after transrectal ultrasound-guided needle biopsy (≥6 cores). To ensure the absence of PCa, men without PCa were only selected if there was no evidence of PCa in a twelve year follow-up period. Samples of men with histologically proven PCa (≥6 core biopsy) were obtained from the Department of Urology at the Erasmus MC, Rotterdam. These consisted of men (n=40) with localized PCa treated with radical prostatectomy and no evidence of biochemical recurrence in the follow-up (minimal 5 years), men with localized PCa treated with radical prostatectomy and biochemical recurrence in the follow-up (n=40), and men with bone and/or lymph metastasis, evidenced by bone scan and/or histologically proven, at the moment of blood draw (n=33). All radical prostatectomy patients reached a PSA nadir of <0.1 ng/ml within three months after surgery. Biochemical recurrence was defined as PSA level ≥0.3 ng/ml. Patient characteristics are depicted in Table 1.

Table 1. Patient characteristics. General characteristics of patients used for a pre-validation of candidate biomarkers. (RP=radical prostatectomy)

	Frequency (n)	Mean age (years) (range)	Median PSA (ng/ml) (range)	Median FU after RP (months) (range)
No-PCa	40	59 (55-66)	0.2 (0.1-0.4)	-
PCa non-recurrence	40	62 (51-74)	6.2 (0.8-65.7)	128 (66-160)
PCa recurrence	40	64 (50-72)	7.9 (0.3-33.1)	140 (70-185)
PCa metastasis	33	68 (50-91)	23.5 (0.1-2550)	-

ELISA for quantification of proteasome subunits

Proteasome concentrations were measured as previously described by Dutaud *et al.* with some minor modifications[134]. Briefly, human or mouse serum (1:20 and 1:10 diluted with PBS containing 0.3% sodium citrate, respectively) or xenograft protein extract was incubated for 1hr on a plate coated with a 1:4,500 dilution of a monoclonal antibody against PSMA1 (clone MCP20, Biomol International). After addition of a 1:1,500 solution of a rabbit anti-proteasome antibody (directed

against beta subunits of the proteasome, PW8155, Biomol International) plates were extensively washed with PBS-Tween buffer. Then, a 1:4,000 solution of HRP conjugated goat anti-rabbit IgG (DakoCytomation) was added and the plate was incubated for 1 hr in the dark. To reveal HRP activity, 50 mM phosphate / 25 mM citrate buffer, pH 5.0 containing 4 mM o-phenylenediamine and 0.0015% H_2O_2 was added to the wells. After 15 min, the reaction was stopped with 2.5 M sulphuric acid. Absorbance values were measured at 492 nm. A standard curve using purified human 20S proteasomes (Biomol International) was calculated using a computer generated 4-PL curve-fit.

ELISA for quantification of ACY1

Human serum (1:20 diluted with PBS containing 0.3% sodium citrate) or 15 times concentrated mouse serum or xenograft protein extract was incubated for 1hr on a plate coated overnight with a 1:500 dilution of a mouse monoclonal antibody against ACY1 (clone 4F1-B7, Abnova, Taipei, Taiwan). Mouse serum (control as well as serum from xenografted mice) was concentrated to enhance the ELISA signal. After addition of a 1:400 solution of a polyclonal antibody (R&D Systems) directed against human ACY1 wells were washed with PBS-Tween buffer. Then, a 1:4,000 solution of HRP conjugated rabbit anti-goat IgG (DakoCytomation) was added and the plate was incubated for 1 hr in the dark. To reveal HRP activity, 50 mM phosphate / 25 mM citrate buffer, pH 5.0 containing 4 mM o-phenylenediamine and 0.0015% H_2O_2 was added to the wells. After 20 min, the reaction was stopped with 2.5 M sulphuric acid. Absorbance values were measured at 492 nm. A standard curve using purified ACY1 antigen (R&D Systems) was calculated using a computer generated 4-PL curve-fit.

Measurement of TYRO3 concentrations

The TYRO3 assay was performed according to the instructions of the manufacturer (R&D Systems Minneapolis, MN, USA). Briefly, 96-well plates were coated overnight with diluted capture antibody. After blocking with PBS and 1% BSA, human (1:20 dilution with 1% BSA/PBS), 15 times concentrated mouse serum samples or xenograft protein extracts were incubated for 2 hours at room temperature. Mouse serum (control as well as serum from xenografted mice) was concentrated to enhance the ELISA signal. After addition of the detection antibody (diluted in 1% BSA/PBS) and Streptavidin-HRP solution, a 1:1 mixture of H_2O_2 and tetramethylbenzidine was added. The optical density of each well was determined at 450 nm, with wavelength correction at 570 nm. A standard curve using purified TYRO3 antigen provided with the assay was calculated using a computer generated 4-PL curve-fit.

Measurement of AXL and MERTK concentrations

The AXL and MERTK assays were performed according to the instructions of the manufacturer (R&D Systems Minneapolis, MN, USA). Briefly, 96-well plates were coated overnight with diluted capture antibody. After blocking with PBS and 1% BSA, human serum samples were 1:20 diluted either in 1% BSA/PBS for the AXL assay or a solution of 1% NP-40, 20 mM Tris, 137 mM NaCl, 10% glycerol, 2 mM EDTA and 1mM activated sodium orthovanadate for the MERTK ELISA. Samples were incubated for 2 hours at room temperature. After addition of the detection antibody (diluted in 1% BSA/PBS or a solution of 20 mM Tris, 137 mM NaCl, 0.05% Tween 20 and 0.1% BSA for the AXL and MERTK assays, respectively) and Streptavidin-HRP solution, a 1:1 mixture of H_2O_2 and tetramethylbenzidine was added. The absorbance of each well was determined at 450 nm, with wavelength correction at 570 nm. A standard curve using purified AXL and MERTK antigen provided with the assay was calculated using a computer generated 4-PL curve-fit.

Statistical analysis

Statistical analyses were performed using the SPSS v15.0 software package (SPSS Inc., Chicago, IL, USA). Differences in variables analysed by ELISA (TYRO3, MERTK, AXL and ACY1) between the various sample groups were examined using the Mann-Whitney U-test with two-sided P values. ROC curves were calculated for TYRO3, MERTK, AXL and ACY1 to assess the diagnostic performances in PCa detection of the various assays.

Results

Proof-of-principle of the experimental setup

When inbred immuno-competent mice are immunized with serum from nude mice bearing human PCa xenografts, antibodies to human tumour antigens should be produced[162]. To test this experimental set-up, western blots loaded with protein extracts of the PC346 or LNCaP PCa cell lines were visualized with preimmune serum or serum from immuno-competent mice immunized with full serum from PC346 and PC339 bearing athymic nude mice (Figure 2). After equal exposure times, several bands emerged in the lanes of the blots developed with immunized mouse serum, in contrast with the blots developed with preimmune serum, indicating an immune response in mice immunized with serum from xenograft-bearing mice.

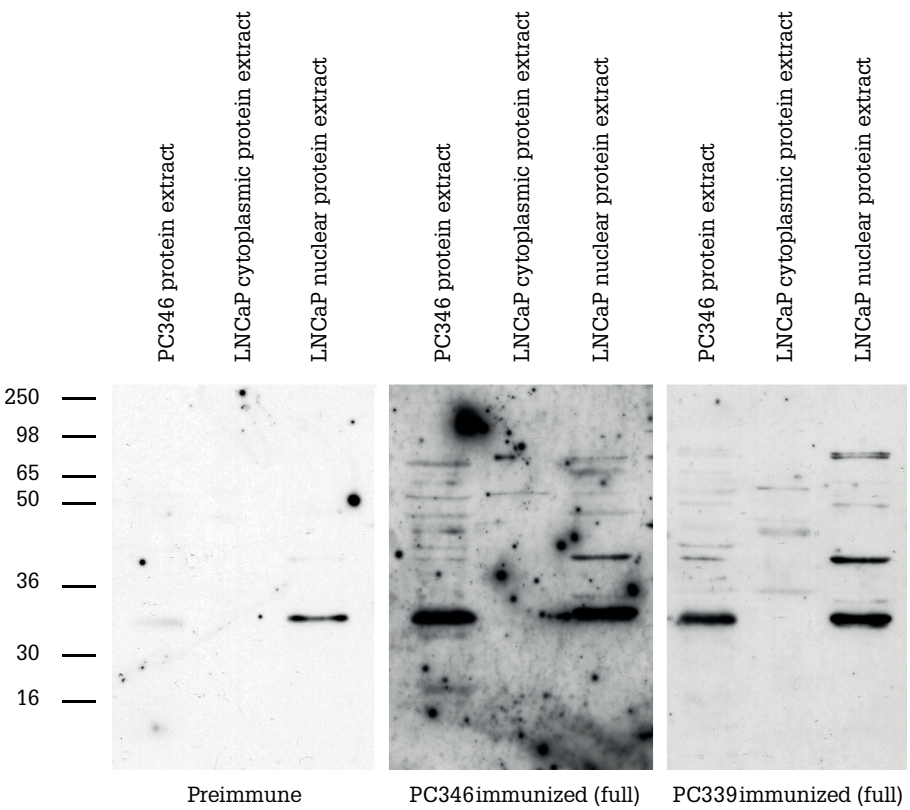


Figure 2. General immune responses in immunized mice. 1D SDS-PAGE western blots loaded with 10µg of PC346 cell line protein and LNCaP cytoplasmic and nuclear protein extracts and developed with preimmune serum or mice immunized with full serum from PC346 and PC339 bearing mice. Blots were exposed for the same amount of time.

For a more specific confirmation of this immune response an assay was developed for the detection of antibodies against PSA. First, PSA concentrations were measured in the serum of mice utilized for the immunization experiment, showing undetectable PSA concentrations in normal nude mice and mice grafted with a PC339 xenograft and high levels of PSA in mice bearing a PC346 xenograft. This is in accordance with the androgen sensitive nature of the PC346 xenograft and androgen unresponsiveness of the PC339 xenograft (see insert Figure 3).
The immunoassay for PSA antibody detection clearly demonstrated the presence of PSA-antibodies in mice immunized with either full serum, serum depleted of high

abundant proteins (depleted serum), or both from PC346-bearing mice (Figure 3). No PSA-antibodies were detected in preimmune serum or in serum from mice immunized with normal mouse serum. No or very low levels of PSA antibodies were detected in mice immunized with either depleted serum, full serum or both from PC339-bearing mice.

Summarizing, these experiments demonstrate that an antibody response against xenograft-derived antigens, such as PSA, is generated in mice immunized with serum from xenograft-bearing mice.

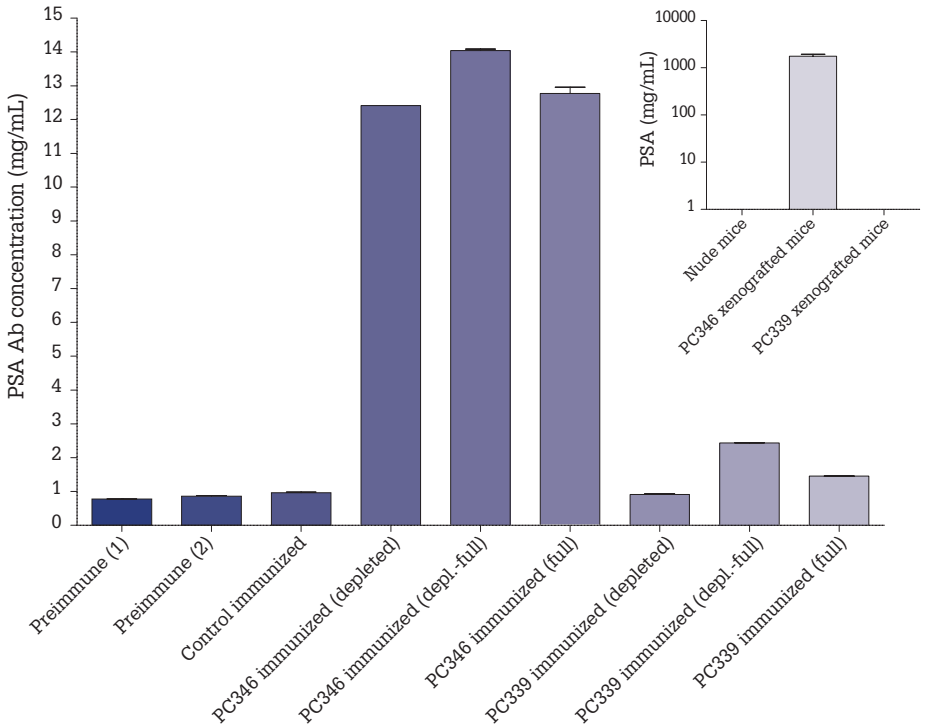


Figure 3. Detection of antibodies directed against PSA. PSA specific antibodies were detected in mice immunized with serum from PC346-bearing mice. No antibodies were detected in preimmune serum and mice immunized with normal mouse serum. PSA antibodies were absent or present in very low amounts in mice immunized with serum from PC339-bearing mice, in accordance with the non-PSA producing nature of this xenograft. Insert; PSA concentrations in serum of normal nude mice and nude mice bearing PC339 or PC346 xenografts. High levels of PSA are detectable in serum of PC346-xenografted mice whereas PSA levels are undetectable in normal nude mice and mice bearing PC339 xenografts.

Identification of antibodies against human PCa-derived proteins

To perform a large-scale identification of antibodies generated against human PCa-derived proteins, sera from mice immunized with either depleted serum, full serum or both from PC346 and PC339-bearing mice as well as preimmune serum and serum from mice immunized with normal mouse serum, were incubated onto ProtoArrays. These ProtoArrays contain approximately 8,000 partial and full-length human proteins. Antibodies bound to spotted proteins were detected using a fluorescent labelled secondary antibody. Processed ProtoArrays showed R^2 >0.96 for duplicated spots across all proteins. 99 Proteins to which an antibody response was observed were identified (Table 2). The fluorescent expression values of the 25 top-ranked identified proteins are depicted in Figure 4.

Table 2. Identified PCa-derived candidate biomarkers. Normalized expression values of identified PCa-derived proteins expressed in immunized mice as identified by ProtoArray analysis (NEV = normalized expression value).

No.	ProtoArray ID	LocusLink ID	Gene name
1	NM_000666.1	95	Aminoacylase 1
2	BC015356.1	5682	Proteasome (prosome, macropain) subunit, alpha type, 1
3	BC008656.1	55812	Spermatogenesis associated 7
4	NM_201262.1	56521	DnaJ (Hsp40) homolog, subfamily C, member 12
5	NM_016497.2	51258	Mitochondrial ribosomal protein L51
6	PV3828	7301	TYRO3 protein tyrosine kinase
7	BC026175.1	1386	Activating transcription factor 2
8	NM_016286.1	51181	Dicarbonyl/L-xylulose reductase
9	NM_004832.1	9446	Glutathione S-transferase omega 1
10	NM_006555.2	10652	YKT6 v-SNARE homolog (S. cerevisiae)
11	NM_004355.1	972	CD74 molecule, major histocompatibility complex, class II invariant chain
12	PV3627	10461	C-mer proto-oncogene tyrosine kinase
13	NM_020525.4	50616	Interleukin 22
14	P2227	5578	Protein kinase C, alpha
15	NM_000594.2	7124	Tumor necrosis factor (TNF superfamily, member 2)
16	XM_378350.2	400027	Hypothetical gene supported by BC047417
17	PV3827	157	Adrenergic, beta, receptor kinase 2
18	PV4131	904	Cyclin T1
19	PV3840	2044	EPH receptor A5
20	BC010537.1	10923	SUB1 homolog (S. cerevisiae)
21	BC017046.1	309	Annexin A6
22	PV3612	6790	Aurora kinase A
23	BC031262.1	1495	Catenin (cadherin-associated protein), alpha 1, 102kDa
24	NM_007194.2	11200	CHK2 checkpoint homolog (S. pombe)
25	NM_007045.2	11116	FGFR1 oncogene partner
26	NM_133443.1	84706	Glutamic pyruvate transaminase (alanine aminotransferase) 2
27	BC008902.2	79571	GRIP and coiled-coil domain containing 1
28	BC029046.1	3005	H1 histone family, member 0
29	NM_020423.1	57147	SCY1-like 3 (S. cerevisiae)
30	NM_018297.2	55768	N-glycanase 1

Gene symbol	Mouse 2nd Ab only (NEV)	preimmune (1) (NEV)	preimmune (2) (NEV)	control immunized (NEV)	PC346 immunized (depleted) (NEV)
ACY1	0.20	1.18	2.12	3.42	21.53
PSMA1	0.23	0.59	0.20	0.51	3.73
SPATA7	0.20	0.31	0.20	0.38	0.76
DNAJC12	0.20	0.77	0.49	0.77	0.85
MRPL51	0.20	0.34	0.20	0.62	1.03
TYRO3	0.20	0.42	0.22	1.33	0.40
ATF2	0.51	1.61	0.20	0.71	2.18
DCXR	0.20	1.06	1.08	0.65	4.43
GSTO1	0.20	0.37	0.24	0.36	0.95
YKT6	0.20	0.55	0.22	1.34	2.38
CD74	0.20	0.62	0.20	0.81	1.05
MERTK	0.20	0.31	0.20	0.35	1.61
IL22	0.20	0.56	4.28	0.57	1.20
PRKCA	0.20	0.53	1.83	2.76	0.22
TNF	0.20	1.78	0.25	0.86	1.87
LOC400027	0.86	1.40	0.20	0.99	1.43
ADRBK2	0.20	0.20	0.20	0.76	0.25
CCNT1	0.47	0.38	1.12	1.67	0.32
EPHA5	0.20	0.78	0.20	0.46	0.58
SUB1	0.20	0.38	0.77	1.20	0.65
ANXA6	0.20	0.78	1.05	3.36	1.87
AURKA	0.44	0.29	3.60	1.32	0.32
CTNNA1	0.21	0.95	0.76	1.65	1.01
CHEK2	0.20	0.24	0.20	0.29	0.52
FGFR1OP	0.23	1.68	0.20	2.63	1.42
GPT2	0.23	0.70	0.99	0.67	1.04
GCC1	0.20	1.05	0.20	1.69	1.34
H1F0	0.20	0.90	1.15	2.54	1.43
SCYL3	0.20	0.45	0.85	0.59	1.10
NGLY1	0.20	0.41	0.20	0.42	0.80

No.	ProtoArray ID	LocusLink ID	Gene name
31	BC053861.1	23133	PHD finger protein 8
32	PV3501	5347	Polo-like kinase 1 (Drosophila)
33	NM_199415.1	22888	FAST kinase domains 5
34	NM_133336.1	7468	Wolf-Hirschhorn syndrome candidate 1
35	BC010125.1	56941	Chromosome 3 open reading frame 37
36	NM_000485.1	353	Adenine phosphoribosyltransferase
37	BC034245.1	55118	Cartilage acidic protein 1
38	PV3367	11200	CHK2 checkpoint homolog (S. pombe)
39	NM_005804.2	10212	DEAD (Asp-Glu-Ala-Asp) box polypeptide 39
40	PV3870	4921	Discoidin domain receptor tyrosine kinase 2
41	BC051695.1	83786	FERM domain containing 8
42	NM_194299.1	221711	Synaptonemal complex protein 2-like
43	NM_002461.1	4597	Mevalonate (diphospho) decarboxylase
44	PV3682	11183	Mitogen-activated protein kinase kinase kinase kinase 5
45	PV3757	85366	Myosin light chain kinase 2
46	NM_005884.2	10298	P21(CDKN1A)-activated kinase 4
47	PV3813	5613	Protein kinase, X-linked
48	NM_006621.3	10768	S-adenosylhomocysteine hydrolase-like 1
49	PV3830	9748	STE20-like kinase (yeast)
50	NM_031472.1	83707	TRNA phosphotransferase 1
51	BC060786.1	55616	Development and differentiation enhancing factor-like 1
52	P3078	7525	V-yes-1 Yamaguchi sarcoma viral oncogene homolog 1
53	NM_001615.2	72	Actin, gamma 2, smooth muscle, enteric
54	NM_152328.3	122622	Adenylosuccinate synthase like 1
55	NM_005738.1	10124	ADP-ribosylation factor-like 4A
56	NM_001124.1	133	Adrenomedullin
57	BC047673.1	63941	N-terminal EF-hand calcium binding protein 3
58	NM_005434.3	7851	Mal, T-cell differentiation protein-like
59	PV3826	1198	CDC-like kinase 3
60	BC059950.1	6136	Ribosomal protein L12
61	NM_152420.1	138199	Chromosome 9 open reading frame 41
62	NM_014183.1	83658	Dynein, light chain, roadblock-type 1
63	PV3689	2045	EPH receptor A7
64	BC052805.1	2039	Erythrocyte membrane protein band 4.9 (dematin)
65	NM_004838.2	9454	Homer homolog 3 (Drosophila)

Gene symbol	Mouse 2nd Ab only (NEV)	preimmune (1) (NEV)	preimmune (2) (NEV)	control immunized (NEV)	PC346 immunized (depleted) (NEV)
PHF8	0.20	0.87	0.20	1.24	1.12
PLK1	0.84	0.43	4.30	0.85	0.40
UBOX5	0.20	0.94	0.20	2.11	1.36
WHSC1	0.20	0.59	3.96	1.33	5.87
C3orf37	2.52	0.20	3.51	0.20	0.22
APRT	0.20	3.14	0.22	0.84	2.00
CRTAC1	0.20	1.33	0.27	2.16	1.33
CHEK2	0.20	0.42	0.33	1.01	0.69
DDX39	0.20	0.76	0.20	1.71	3.29
DDR2	0.20	0.81	0.53	0.50	0.99
FRMD8	0.20	0.20	3.07	0.20	0.22
SYCP2L	0.20	0.92	0.35	1.30	1.55
MVD	0.20	1.06	0.91	0.97	1.44
MAP4K5	0.20	0.29	0.36	1.31	0.38
MYLK2	0.65	1.73	0.20	1.37	1.63
PAK4	0.20	0.64	2.22	1.41	1.21
PRKX	0.22	2.04	4.08	2.06	6.41
AHCYL1	0.20	0.80	1.52	0.20	0.22
SLK	0.23	0.75	0.91	3.10	0.75
TRPT1	0.51	0.87	3.61	0.61	1.16
DDEFL1	0.20	0.90	0.20	1.00	1.28
YES1	0.20	1.29	0.20	2.29	1.49
ACTG2	0.20	0.86	0.46	0.95	1.68
ADSSL1	0.20	0.20	1.77	0.20	0.22
ARL4A	0.20	0.43	0.27	0.87	0.74
ADM	0.20	0.80	0.43	1.01	1.53
NECAB3	0.20	0.20	2.02	0.20	0.22
MALL	0.20	0.36	2.41	0.44	0.73
CLK3	0.20	1.29	0.82	1.62	1.23
RPL12	0.23	0.31	1.48	0.83	0.86
C9orf41	0.21	0.97	1.31	1.84	1.77
DYNLRB1	0.57	0.85	0.55	0.72	1.26
EPHA7	1.16	0.75	1.58	1.18	1.19
EPB49	0.78	1.18	3.22	0.58	2.03
HOMER3	0.20	1.57	0.90	0.96	1.59

No.	ProtoArray ID	LocusLink ID	Gene name
66	NM_139016.2	25943	Chromosome 20 open reading frame 194
67	BC012131.1	112849	Chromosome 14 open reading frame 149
68	BC033196.1	283229	EF-hand calcium binding domain 4A
69	BC008200.1	3856	Keratin 8
70	NM_018396.1	55798	Methyltransferase like 2B
71	BC031469.1	554207	Hypothetical LOC554207
72	PV3835	4638	Myosin light chain kinase
73	NM_017506.1	26659	Olfactory receptor, family 7, subfamily A, member 5
74	NM_006695.2	10900	RUN domain containing 3A
75	NM_198395.1	10146	GTPase activating protein (SH3 domain) binding protein 1
76	BC020726.1	8796	Sciellin
77	NM_004169.2	6470	Serine hydroxymethyltransferase 1 (soluble)
78	BC032825.2	6456	SH3-domain GRB2-like 2
79	BC018122.1	7296	Thioredoxin reductase 1
80	NM_003289.3	7169	Tropomyosin 2 (beta)
81	PV3792	7272	TTK protein kinase
82	PV3869	3815	V-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
83	P2782	7535	Zeta-chain (TCR) associated protein kinase 70kDa
84	BC004207.1	11200	CHK2 checkpoint homolog (S. pombe)
85	BC002448.2	3983	Actin binding LIM protein 1
86	BC058926.1	1499	Catenin (cadherin-associated protein), beta 1, 88kDa
87	NM_001277.1	1119	Choline kinase alpha
88	NM_080664.1	112487	Chromosome 14 open reading frame 126
89	BC023567.2	51379	Cytokine receptor-like factor 3
90	PV3354	2242	Feline sarcoma oncogene
91	BC001772.1	5859	Glutaminyl-tRNA synthetase
92	NM_018090.2	55707	NECAP endocytosis associated 2
93	BC031074.1	54956	Poly (ADP-ribose) polymerase family, member 16
94	BC017066.1	133619	Proline-rich coiled-coil 1
95	NM_152646.2	196872	Hypothetical LOC196872
96	NM_005550.2	3801	Kinesin family member C3
97	PV3370	4145	Megakaryocyte-associated tyrosine kinase
98	NM_005371.2	4234	Methyltransferase like 1
99	NM_002444.1	4478	Moesin

Gene symbol	Mouse 2nd Ab only (NEV)	preimmune (1) (NEV)	preimmune (2) (NEV)	control immunized (NEV)	PC346 immunized (depleted) (NEV)
C20orf194	0.28	0.20	2.16	0.20	0.22
C14orf149	0.20	0.44	0.83	0.66	1.11
EFCAB4A	0.20	1.79	2.02	1.31	2.61
KRT8	1.11	1.18	1.74	1.11	1.69
METTL2B	0.20	0.63	0.39	0.57	0.91
LOC554207	0.28	1.26	0.30	1.25	1.67
MYLK	0.45	0.67	0.20	2.27	0.74
OR7A5	0.20	0.49	0.63	1.45	1.20
RUNDC3A	0.21	1.54	1.26	1.23	1.15
G3BP1	1.09	0.89	1.15	3.03	1.48
SCEL	1.46	1.18	4.60	2.60	1.61
SHMT1	0.20	0.41	1.48	0.60	0.96
SH3GL2	0.79	0.20	3.20	0.20	0.22
TXNRD1	0.20	0.85	1.24	1.50	1.80
TPM2	0.20	0.20	2.56	0.20	0.22
TTK	0.38	1.36	2.13	1.88	0.76
KIT	0.25	0.55	0.39	1.94	0.42
ZAP70	0.22	0.57	3.36	1.46	0.36
CHEK2	0.20	0.40	0.93	0.61	1.29
ABLIM1	2.16	1.21	2.01	1.49	1.39
CTNNB1	0.20	1.40	0.46	1.36	1.72
CHKA	0.28	0.94	2.43	1.95	2.52
C14orf126	0.54	0.73	4.09	2.32	1.38
CRLF3	0.20	1.33	0.78	2.36	1.62
FES	0.32	0.71	1.82	2.54	0.93
QARS	0.24	0.56	0.79	0.36	0.90
NECAP2	0.20	0.96	2.23	0.90	1.26
PARP16	0.20	0.65	0.88	1.63	0.97
PRRC1	0.20	0.90	1.46	0.51	1.16
MGC23270	0.86	2.49	3.13	0.81	2.66
KIFC3	1.06	0.56	3.94	3.36	2.01
MATK	0.20	0.62	0.55	0.79	0.77
METTL1	0.20	0.52	0.64	1.13	1.21
MSN	0.20	1.56	0.44	1.47	2.68

Table 2, continued.

No.	PC346 immunized (depleted - full) (NEV)	PC346 immunized (full) (NEV)	PC339 immunized (depleted) (NEV)	PC339 immunized (depleted - full) (NEV)	PC339 immunized (full) (NEV)
1	474.53	63.73	17.25	114.17	10.90
2	315.90	2.04	1.40	2.57	0.65
3	1.95	0.77	0.59	2.03	0.59
4	7.40	33.48	0.95	2.13	1.15
5	1.66	0.95	0.87	10.31	0.60
6	0.37	2.54	0.83	2.02	5.85
7	7.37	1.77	1.10	0.27	0.85
8	189.26	4.59	1.57	1.29	0.88
9	2.10	0.56	1.71	79.66	0.78
10	0.77	1.87	2.35	1.60	18.55
11	0.31	3.62	0.95	0.35	1.48
12	2.45	0.56	0.20	2.60	0.36
13	5.94	8.87	0.96	2.38	5.37
14	2.62	15.16	0.75	0.63	9.20
15	1.02	1.11	12.36	0.29	1.02
16	0.31	4.32	1.52	0.55	3.27
17	0.31	3.88	0.20	0.20	1.79
18	1.47	5.91	0.59	0.97	4.85
19	0.31	6.56	0.55	0.20	1.14
20	1.42	5.07	0.49	0.28	3.39
21	9.81	23.47	1.63	0.61	1.39
22	5.16	1.53	0.20	1.05	13.11
23	0.31	5.41	1.28	0.86	9.58
24	0.34	18.14	0.34	0.49	0.48
25	0.31	3.28	1.55	0.29	2.58
26	1.88	4.41	1.11	0.41	2.21
27	0.82	1.72	2.76	0.27	1.10
28	2.23	8.25	1.43	0.37	7.57
29	6.00	1.25	1.35	1.23	0.60
30	0.31	1.16	0.67	19.15	0.44
31	0.31	1.45	1.96	1.56	0.96
32	6.34	2.65	0.40	1.81	2.17

	Location	Type	Prior linked to PCa	Suggested as PCa biomarker
	cytoplasm	peptidase	yes[165]	yes[165]
	cytoplasm	peptidase	no	no
	unknown	other	no	no
	unknown	other	Yes[166]	no
	cytoplasm	other	no	no
	plasma membrane	kinase	no	no
	nucleus	transcription regulator	yes[167]	no
	cytoplasm	enzyme	yes[168]	yes[168]
	cytoplasm	enzyme	yes[169]	no
	cytoplasm	enzyme	no	no
	plasma membrane	transmembrane receptor	yes[170]	no
	plasma membrane	kinase	yes[171]	no
	extracellular space	cytokine	no	no
	cytoplasm	kinase	yes[172]	no
	extracellular space	cytokine	yes[173]	yes[174]
	unknown	other	no	no
	cytoplasm	kinase	no	no
	nucleus	transcription regulator	no	no
	plasma membrane	kinase	no	no
	nucleus	transcription regulator	no	no
	plasma membrane	other	no	no
	nucleus	kinase	yes[175]	no
	plasma membrane	other	yes[176]	yes[177]
	nucleus	kinase	yes[178]	no
	cytoplasm	other	no	no
	unknown	enzyme	no	no
	cytoplasm	other	no	no
	nucleus	other	no	no
	cytoplasm	kinase	no	no
	cytoplasm	enzyme	no	no
	unknown	other	no	no
	nucleus	kinase	yes[179]	yes[179]

No.	PC346 immunized (depleted - full) (NEV)	PC346 immunized (full) (NEV)	PC339 immunized (depleted) (NEV)	PC339 immunized (depleted - full) (NEV)	PC339 immunized (full) (NEV)
33	0.33	4.21	1.80	0.32	1.73
34	6.09	6.23	1.93	0.44	2.02
35	4.90	0.23	0.20	3.19	1.87
36	0.99	1.51	2.79	0.32	1.13
37	0.84	1.41	2.79	0.20	1.05
38	0.38	39.30	0.35	0.29	1.30
39	1.18	0.63	1.19	0.26	0.82
40	6.32	2.20	0.62	0.22	1.87
41	13.23	0.23	0.20	1.15	0.20
42	1.13	2.68	1.41	0.38	2.56
43	1.68	1.36	7.18	0.95	1.33
44	1.24	2.03	0.20	0.42	2.66
45	0.31	2.95	1.30	0.20	3.64
46	21.28	6.36	0.74	0.90	1.48
47	2.70	9.88	1.53	9.87	8.00
48	1.48	0.23	0.20	0.63	3.85
49	0.98	3.75	3.92	0.63	3.20
50	5.69	1.61	0.97	2.05	4.01
51	0.85	1.50	2.80	0.21	1.19
52	0.31	5.38	0.85	0.20	3.34
53	1.98	0.78	3.40	0.42	0.86
54	2.15	0.23	0.20	1.75	0.20
55	0.56	5.18	0.66	0.20	0.62
56	2.03	1.25	2.86	0.40	1.42
57	1.54	0.23	0.20	20.11	0.20
58	4.85	2.81	0.65	0.50	0.64
59	1.17	7.73	1.30	0.23	0.99
60	15.07	1.42	0.63	0.52	1.25
61	2.34	11.87	1.39	0.90	2.46
62	0.89	1.68	3.02	0.28	0.87
63	1.69	7.24	0.64	0.73	1.20
64	3.73	4.04	1.49	1.20	2.74
65	2.53	10.58	2.76	0.79	1.99
66	6.04	0.23	0.20	0.87	0.20

Location	Type	Prior linked to PCa	Suggested as PCa biomarker
nucleus	other	no	no
nucleus	other	no	no
unknown	other	no	no
cytoplasm	enzyme	no	no
extracellular space	other	no	no
nucleus	kinase	yes[178]	no
nucleus	enzyme	no	no
plasma membrane	kinase	no	no
unknown	other	no	no
unknown	other	no	no
cytoplasm	enzyme	no	no
cytoplasm	kinase	no	no
cytoplasm	kinase	no	no
cytoplasm	kinase	yes[180]	no
cytoplasm	kinase	no	no
cytoplasm	enzyme	no	no
nucleus	kinase	no	no
unknown	enzyme	no	no
unknown	other	no	no
cytoplasm	kinase	no	no
cytoplasm	other	yes[181]	no
cytoplasm	enzyme	no	no
nucleus	enzyme	no	no
extracellular space	other	yes[182]	no
cytoplasm	other	no	no
plasma membrane	other	no	no
nucleus	kinase	yes[183]	no
cytoplasm	other	no	no
unknown	other	no	no
cytoplasm	other	yes[184]	no
plasma membrane	kinase	yes[185]	no
plasma membrane	other	yes[186]	no
plasma membrane	other	no	no
nucleus	other	no	no

No.	PC346 immunized (depleted - full) (NEV)	PC346 immunized (full) (NEV)	PC339 immunized (depleted) (NEV)	PC339 immunized (depleted - full) (NEV)	PC339 immunized (full) (NEV)
67	1.74	1.29	1.00	0.42	2.81
68	4.55	6.31	1.12	3.23	5.14
69	3.24	13.41	1.53	0.47	1.46
70	0.83	1.17	0.90	0.33	28.95
71	0.31	2.32	1.35	1.36	0.88
72	0.31	4.22	0.42	0.20	1.43
73	1.49	0.93	0.95	0.37	10.01
74	1.47	1.32	5.70	0.38	0.97
75	1.83	3.55	2.23	2.62	3.78
76	7.25	7.48	1.56	2.83	8.29
77	4.95	1.53	0.73	1.45	1.13
78	5.59	0.23	0.20	1.21	0.20
79	14.15	2.31	1.42	1.60	1.57
80	2.91	0.23	0.20	2.75	0.20
81	2.54	31.31	1.77	2.07	3.53
82	0.70	3.58	0.52	0.20	3.05
83	25.50	1.69	0.24	1.26	1.46
84	1.22	11.15	0.99	0.25	0.72
85	3.14	8.23	1.09	0.76	2.19
86	15.97	1.75	1.25	0.45	1.34
87	22.35	2.18	1.29	0.42	1.82
88	6.72	1.31	1.09	4.00	1.50
89	1.25	1.61	1.75	0.48	7.13
90	1.87	3.63	0.48	0.35	7.62
91	6.71	1.50	0.55	0.46	0.38
92	2.29	1.87	6.92	0.87	2.30
93	1.99	1.51	1.31	0.87	3.68
94	1.76	2.22	0.86	1.66	1.79
95	6.52	2.59	0.88	1.49	5.03
96	5.47	4.82	2.10	2.30	2.13
97	0.31	3.56	0.63	0.49	1.26
98	1.39	3.46	0.98	0.43	0.83
99	0.31	4.14	1.27	0.47	1.79

	Location	Type	Prior linked to PCa	Suggested as PCa biomarker
	unknown	other	no	no
	unknown	other	no	no
	cytoplasm	kinase	yes[187]	yes[187]
	unknown	enzyme	no	no
			no	no
	cytoplasm	kinase	no	no
	plasma membrane	other	no	no
	cytoplasm	other	no	no
	nucleus	enzyme	no	no
	cytoplasm	other	no	no
	cytoplasm	enzyme	no	no
	plasma membrane	enzyme	no	no
	cytoplasm	enzyme	no	no
	cytoplasm	other	no	no
	nucleus	kinase	no	no
	plasma membrane	kinase	yes[188]	yes[189]
	plasma membrane	kinase	no	no
	nucleus	kinase	yes[178]	no
	cytoplasm	other	no	no
	nucleus	transcription regulator	yes[190]	no
	cytoplasm	kinase	no	no
	unknown	other	no	no
	unknown	other	no	no
	cytoplasm	kinase	no	no
	cytoplasm	enzyme	no	no
	cytoplasm	other	no	no
	unknown	other	no	no
	unknown	other	no	no
	unknown	other	no	no
	cytoplasm	enzyme	no	no
			no	no
	unknown	enzyme	no	no
	plasma membrane	other	yes[191]	no

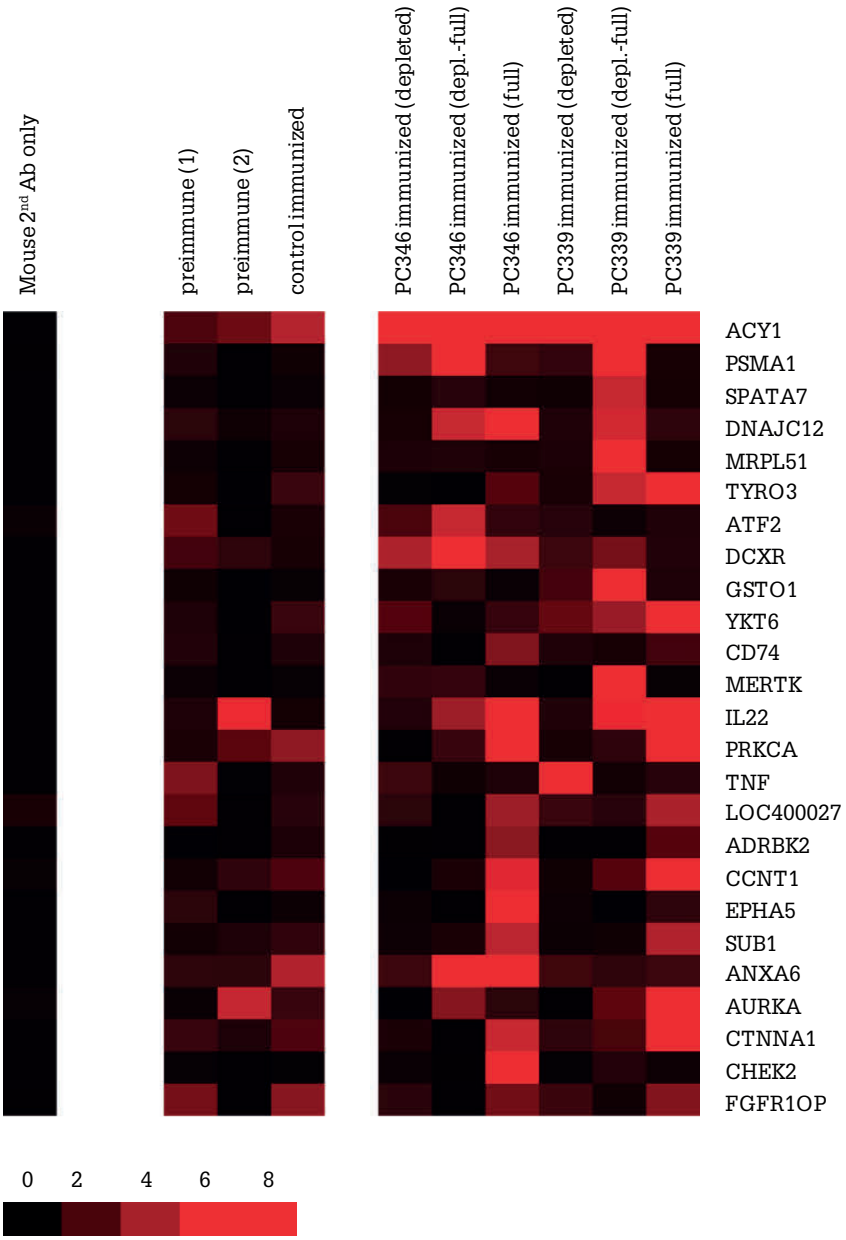


Figure 4. Top-ranked ProtoArray-identified proteins.
Heatmap of the top-25 PCa-derived proteins identified in immunized mice by ProtoArray analysis. A full overview of all identified proteins is depicted in Table 2.

Validation of identified PCa-derived antigens in serum of xenograft-bearing mice

A second confirmation of the experimental setup consisted of the quantification of identified proteins in the serum of nude athymic mice, mice bearing a PC346 or PC339 xenograft and in the xenografted tissue. For this purpose, immunoassays were developed to estimate levels of the two proteins with the highest ProtoArray expression in immunized mice, aminoacylase 1 (ACY1) and proteasome subunit alpha type 1 (PSMA1). A commercial immunoassay was obtained to measure levels of another high-ranked candidate, TYRO3 tyrosine protein kinase (TYRO3), a member of the TAM family of receptor tyrosine kinases (RTKs) (Figure 4). Figure 5a shows that ACY1 is detectable in concentrated serum of PC346 and PC339 harbouring mice and was absent in the concentrated serum of normal non-xenografted nude mice. In addition, ACY1 was detected in both xenograft protein extracts. The protein with the second highest ProtoArray expression, PSMA1, was present in the serum of both xenograft-bearing mice as well as in both xenograft protein extracts, while absent in the serum of normal nude mice (Figure 5b). TYRO3 was detected in the concentrated serum of PC346 xenograft-bearing mice and the protein extracts of both xenografts but absent in the concentrated serum of normal non-xenografted nude mice and mice harbouring a PC339 xenograft (Figure 5c). Hence, the detection of ACY1, PSMA1 and TYRO3 in serum of xenograft-bearing mice confirms their identification by ProtoArray analysis and provides a second validation of the experimental setup.

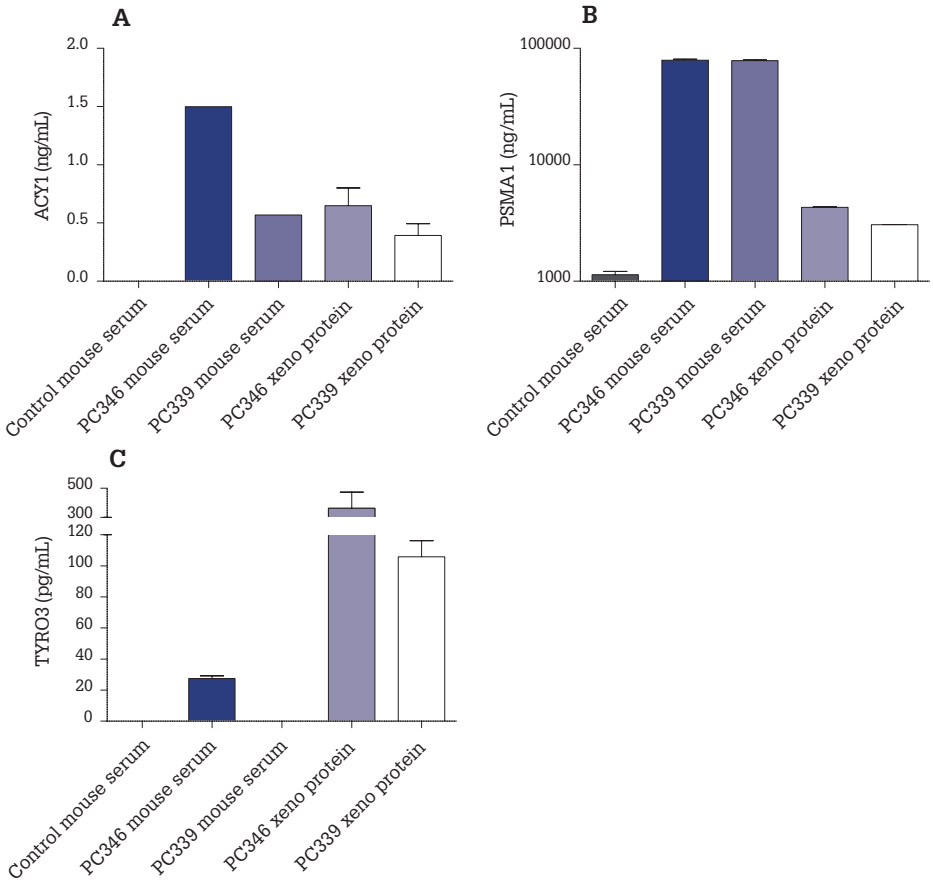
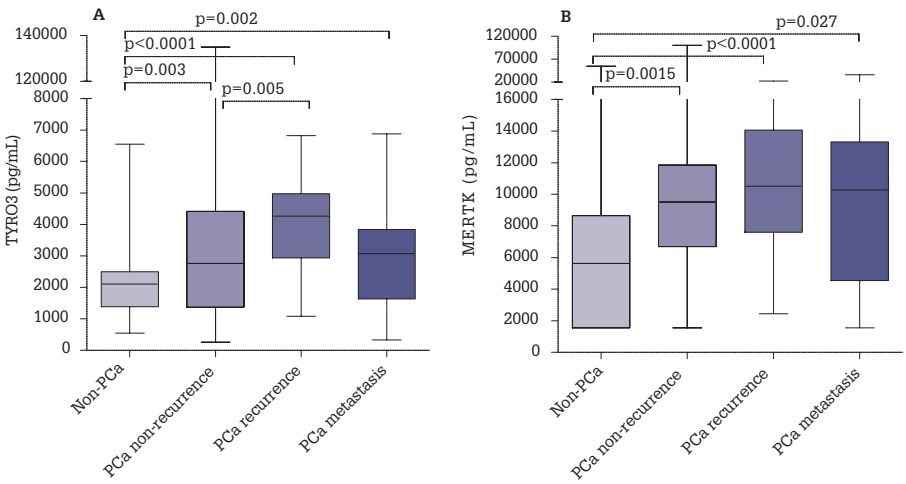


Figure 5. Expression of identified antigens in xenograft-bearing mice. Mean concentrations (± standard error of the mean) of ACY1 (panel A), PSMA1 (panel B) and TYRO3 (panel C) in serum of nude mice, serum of mice bearing a PC346 or PC339 xenograft and PC346 and PC339 xenograft protein extracts.

Pre-validation of identified candidate biomarkers in human serum samples

Concentrations of several identified candidate biomarkers were determined in sera of men with and without PCa. Since ACY1, PSMA1 and TYRO3 were detected in the serum of xenograft bearing mice, these were selected for initial validation. In addition, the two other members of the TAM receptor family, c-mer proto-oncogene tyrosine kinase (MERTK), and AXL receptor tyrosine kinase (AXL) were added, as MERTK was also among the high-ranked proteins identified by ProtoArray analysis. For validation, serum samples of men without PCa (n=40), men undergoing a radical prostatectomy for PCa with and without recurrence in the follow-up (n=80), and men with PCa metastasis (n=33) were retrospectively collected (Table 1). For TYRO3 and MERTK, serum concentrations were significantly increased in men with localized or metastasized PCa compared to men without PCa (Figures 6a and 6b). In contrast, serum concentrations of AXL were significantly lower in men with localized PCa, compared to men without PCa or PCa metastasis (Figure 6c). ACY1 serum levels were significantly higher in patients with PCa metastasis compared to patients with localized PCa or men without PCa (Figure 6d). PSMA1 concentrations did not differ significantly between the various groups (Figure 6e). To assess the value of the candidate markers in PCa detection, receiver operating characteristics (ROC) curves were generated for TYRO3, MERTK, AXL and ACY1, based upon analysis of men without PCa and men with localized PCa (Figure 7 and Table 3).

To summarize, these data show that several antigens to which an antibody response was identified in the xenograft mouse model also were differentially expressed in men with and without PCa.



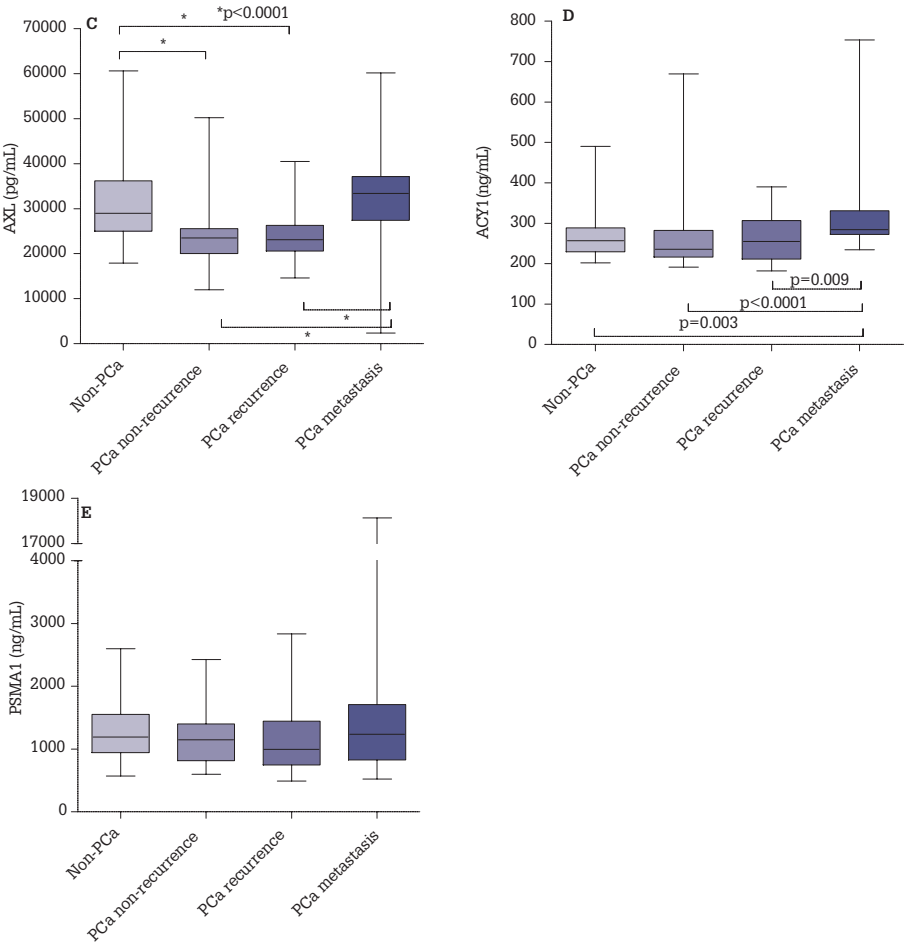


Figure 6. Expression of discovered PCa xenograft-derived antigens in human serum. Box plots of serum concentrations of TYRO3 (panel A), MERTK (panel B), AXL (panel C), ACY1 (panel D) and PSMA1 (panel E) in men without PCA, men with PCa without recurrence, men with PCa and recurrence and men with PCa metastasis. (Boxes, interquartile range, containing 50% of values; whiskers extending from the highest to lowest values; line across box, median).

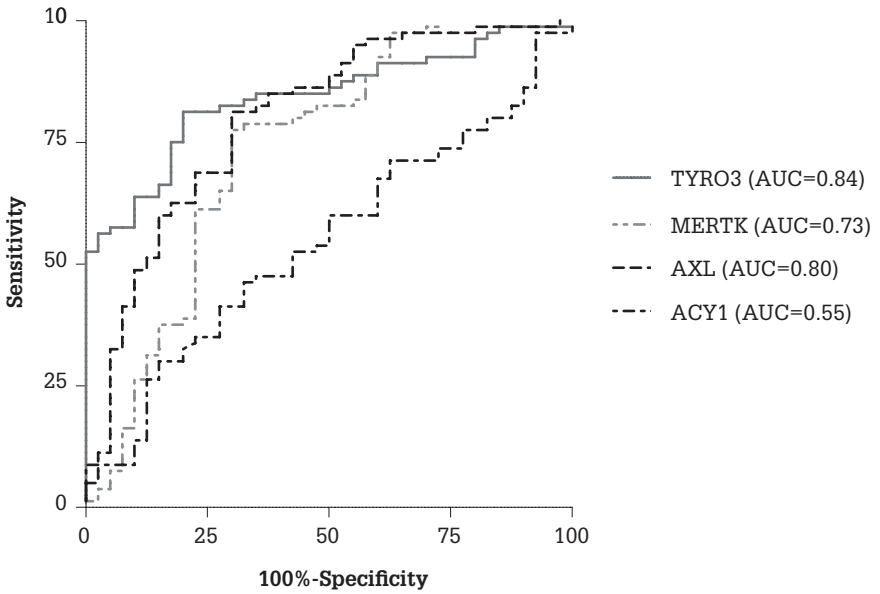


Figure 7. Predictive value of identified candidate biomarkers in PCa detection. ROC curves of TYRO3, MERTK, AXL and ACY1 based on men without PCa and the combined groups of men with localized PCa. Detailed information on the areas under the curve (AUCs) is depicted in Table 3.

Table 3. Backward stepwise logistic regression model of identified candidate biomarkers in PCa. Areas under the curve (AUCs) of TYRO3, MERTK, AXL and ACY1.

	AUC (95% CI)	p-value
TYRO3	0.84 (0.77-0.91)	<0.0001
MERTK	0.73 (0.63-0.84)	<0.0001
AXL	0.80 (0.71-0.89)	<0.0001
ACY1	0.55 (0.44-0.65)	0.41

Discussion

The present study describes a unique xenograft-based biomarker discovery platform and proves its usefulness in the discovery of novel serum markers for PCa. By immunizing immuno-competent mice with serum from nude mice bearing PCa xenografts, an antibody response against xenograft-derived antigens was elicited. By probing protein microarrays with serum from immunized mice, several PCa-derived antigens were identified, of which a subset was successfully retrieved in serum from mice bearing PCa xenografts (Figure 5) and validated in human serum samples (Figure 6).

A literature search learned that the majority of identified proteins have not been previously reported in relation with PCa, although some proteins have been proposed as possible markers (Table 2). Furthermore, the validated candidate biomarkers have been shown to be present in mouse and human sera in the ng/ml and pg/mL ranges, which is in the same serologic detection range as PSA, making them feasible for diagnostic purposes and confirming the ability of this biomarker discovery platform to detect the low abundant proteins. To exclude the presence of false-positive results due to possible background noise in the present ProtoArray and immunoassay based approaches, we performed several control experiments, including samples without the antigen, with standard series of antigen and preimmune serum hybridizations. In addition, a ProtoArray was developed with secondary antibody only, showing only a few protein spots that were quite strongly recognized by the secondary antibody.

Due to the heterogenic nature of PCa, a multitude of different PCa xenografts would be required to identify markers for each patient subpopulation. For this reason we utilized two different xenografts in this study, androgen sensitive PC346 and androgen unresponsive PC339, representing early and late stage disease, from which we indeed identified differences in antigens (Figure 4 and Table 2). Besides analysis of xenografts from different origin, also tumour location (e.g. subcutaneous, orthotopic or bone metastasis) might result in differential xenograft protein expression and can be studied using our approach.

Distinct differences are seen in detected xenograft-derived antigens between mice immunized with either full or depleted sera (Figure 4). Only for ACY1, a ProtoArray response is seen in all samples, while the antibody responses after immunization with either full or depleted serum seem exclusive. Overall, immunization with depleted serum showed the least pronounced ProtoArray responses.

This variability can be attributed to the individual antibody-responses between the immuno-competent mice, as is well-established[192-194]. Secondly, differences in antibody responses may be attributable to the use of either full or depleted serum.

After depletion of high abundant proteins, also low-molecular-weight proteins are removed, as albumin and immunoglobulin G function as carrier proteins[195,196].
Overexpression of TAM receptors has been reported for a wide spectrum of cancers. With respect to PCa, different expression patterns of AXL and MERTK have been reported[171,197-199]. Differences in expression patterns were also observed in the serum concentrations of the TAM receptors in men with and without PCa in the present study (Figure 5), strengthened by data extracted from Oncomine, showing an increased gene expression of MERTK in PCa tissue compared to normal prostate tissue, while gene expression of AXL shows the opposite (Figure 8)[200].

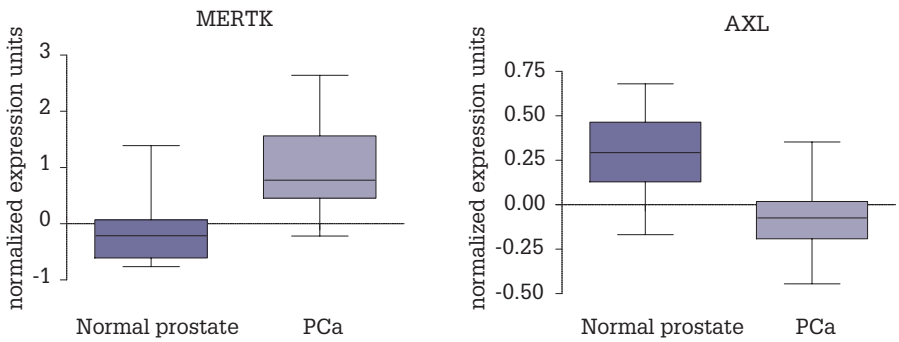


Figure 8. Expression of MERTK and AXL in normal and PCa tissue. Oncomine gene expression results of TAM receptors over- or underexpressed in normal prostate compared to PCa tissue showing opposite patterns for MERTK and AXL. Significant results regarding TYRO3 expression in normal compared to malignant prostate tissue are not available. (P-value threshold: 1E-4; Outlier rank threshold: 50)

Recently, the proteasome β6 subunit was identified in serum from PCa patients[141]. The contrasting finding of high concentrations in serum xenograft-bearing mice and the absence of increased PSMA1 concentrations in men with PCa may be explained by the fact that the amount of released proteasomes from PCa cells does not significantly contribute to the total amount of proteasomes circulating under normal physiological conditions.
ACY1 has been detected as overexpressed in PCa needle biopsy specimens[165]. The present study showed that ACY1 concentrations were significantly higher in men with PCa metastasis compared to men without PCa or with localized PCa. ACY1 may thus function as a potential marker for patients suspected of having disseminated disease.

To conclude, this study shows that by utilizing a unique xenograft-based immunization method it is possible to detect novel PCa biomarkers. The same approach could be taken for any human cancer, greatly assisting current biomarker discovery. The initial results of TYRO3, MERTK and AXL with respect to PCa detection are promising, although further validation of these and other candidate biomarkers in multi-center series is necessary.

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CHAPTER 4

PROSTATE-SPECIFIC ANTIGEN ISOFORM P2PSA IN COMBINATION WITH TOTAL PSA AND FREE PSA IMPROVES DIAGNOSTIC ACCURACY IN PROSTATE CANCER DETECTION

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Abstract

Background: Novel markers for prostate cancer (PCa) detection are needed. Total prostate-specific antigen (tPSA) and percent free prostate-specific antigen (%fPSA = tPSA/fPSA) lack diagnostic specificity.
Objective: To evaluate the use of prostate-specific antigen (PSA) isoforms p2PSA and benign prostatic hyperplasia–associated PSA (BPHA).
Design, setting, and participants: Our study included 405 serum samples from the Rotterdam arm of the European Randomised Study of Screening for Prostate Cancer and 351 samples from the Urology Department of Innsbruck Medical University.
Measurements: BPHA, tPSA, fPSA, and p2PSA levels were measured by Beckman-Coulter Access Immunoassay. In addition, the Beckman Coulter Prostate Health Index was calculated: phi = (p2PSA/fPSA) x √(tPSA).
Results and limitations: The p2PSA and phi levels differed significantly between men with and without PCa. No difference in BPHA levels was observed. The highest PCa predictive value in both cohorts was achieved by phi with areas under the curve (AUCs) of 0.750 and 0.709, a significant increase compared to tPSA (AUC: 0.585 and 0.534) and %fPSA (AUC: 0.675 and 0.576). Also, %p2PSA (p2PSA/fPSA) showed significantly higher AUCs compared to tPSA and %fPSA (AUC: 0.716 and 0.695, respectively). At 95% and 90% sensitivity, the specificities of phi were 23% and 31% compared to 10% and 8% for tPSA, respectively. In both cohorts, multivariate analysis showed a significant increase in PCa predictive value after addition of p2PSA to a model consisting of tPSA and fPSA (increase in AUC from 0.675 to 0.755 and from 0.581 to 0.697, respectively). Additionally, the specificity at 95% sensitivity increased from 8% to 24% and 7% to 23%, respectively. Furthermore, %p2PSA, phi, and the model consisting of tPSA and fPSA with or without the addition of p2PSA missed the least of the tumours with a biopsy or pathologic Gleason score ≥7 at 95% and 90% sensitivity.
Conclusions: This study shows significant increases in PCa predictive value and specificity of phi and %p2PSA compared to tPSA and %fPSA. p2PSA has limited additional value in identifying aggressive PCa (Gleason score ≥7).

Introduction

Because serum total prostate-specific antigen (tPSA) and percent free prostate-specific antigen (%fPSA) lack clear thresholds balancing specificity and sensitivity for the early detection of prostate cancer (PCa), continuous efforts are being made to discover new PCa markers. Detailed examination of the free prostate-specific

antigen (fPSA) fraction resulted in the identification of several distinctive fPSA forms, among which a mixture of precursor isoforms of prostate-specific antigen (pPSA or proPSA) and a form designated ''benign'' PSA (i.e., benign prostatic hyperplasia–associated PSA [BPHA])[52-54,58,59,201]. A study specifically concentrating on the precursor isoform of PSA containing two amino acids in the propeptide leader confirmed the presence of [-2]proPSA in serum of men with PCa, in which [-2]proPSA formed 25–95% of the fPSA fraction, in contrast with 6–19% in biopsy-negative men[201]. Initial reports investigating the clinical value of [-2]proPSA in screening for PCa showed that [-2]proPSA serum concentrations were in general higher in men with PCa compared to men without cancer[61-64]. Recently, reports by Sokoll *et al.*[202] and Stephan *et al.*[203] showed that [-2]proPSA is able to significantly improve PCa detection. Characterisation of BPHA showed that it contains 237 amino acids like PSA but is clipped at several specific amino acid residues[58]. Measurement of BPHA concentrations in serum demonstrated that BPHA represented 25% of the fPSA in biopsy-negative men and was significantly higher in benign compared to PCa serum[59]. Furthermore, BPHA outperformed fPSA as well as tPSA in the prediction of transition zone enlargement[60]. A recent study evaluating the use of BPHA in discriminating PCa patients from patients without evidence of PCa showed that BPHA might improve PCa detection[204]. Results on a possible prognostic role for BPHA as well as [-2]proPSA have proven inconclusive[81,82,203,204]. In this two-centre study, both p2PSA and BPHA serum concentrations were measured in men with and without PCa to evaluate the additional clinical utility of p2PSA and BPHA next to tPSA and fPSA in a screening setting for PCa. (Note that the analyte is referred to as [-2]proPSA and the assay as p2PSA; for simplicity, p2PSA is used throughout the remainder of this report.) Also, several calculated derivatives, among which the Beckman Coulter Prostate Health Index (phi), a mathematical combination of PSA, fPSA, and p2PSA, were assessed. In addition, all analysed variables were assessed with respect to biopsy and pathologic Gleason scores and their ability to detect cancers with a Gleason score of ≥7.

Patients and Methods

Patient selection

Serum samples from site 1 were obtained from the biorepository of the Rotterdam arm of the European Study of Screening for Prostate Cancer. From 1994 to February 1997, indications for a prostate biopsy consisted of a tPSA level >4.0 ng/ml, abnormal digital rectal examination (DRE), or abnormal transrectal ultrasound (TRUS). In 1997, this combination was replaced by PSA testing only[205]. Serum samples from site 2 were

enrolled from the PCa screening study at the Urology Department of the University of Innsbruck, which started in 1993. Indications for biopsy were based upon cancer probabilities estimated by an artificial neural network including tPSA, fPSA, age, DRE, and TRUS[206]. In addition, the concept of PSA velocity was incorporated in the diagnostic evaluation in 2005[206]. Only screen-detected samples of men with and without PCa were included in the present study. Inclusion criteria were age ≥50 yr, a tPSA concentration of 2–10 ng/ ml, a performed TRUS-guided needle biopsy (six or more cores), and a histologically confirmed diagnosis. Exclusion criteria were a prior history of PCa, acute prostatitis, urinary tract infection, prior transurethral resection of the prostate, and use of drugs or other therapies within 6 wk preceding blood draw that might influence PSA concentration. Blood specimens were processed and refrigerated within 3 h of blood draw and stored at -80 °C. A total of 405 men could be enrolled at site 1 (226 PCa cases, 179 non-PCa cases) and a total of 351 men at site 2 (174 PCa cases, 177 non-PCa cases).

Measurement of tPSA, fPSA, p2PSA, and BPHA serum concentrations

At both sites, archival serum samples were analysed for tPSA, fPSA, p2PSA, and BPHA on the Access 2 Immunoassay System (Beckman Coulter, Brea, CA, USA) using the following serum assays: the US Food and Drug Association–approved Access Hybritech PSA and Access Hybritech free PSA; Access BPHA, which measures BPHA; and p2PSA, which measures p2PSA (*the latter two for research use only, not for patient management*). All assays were performed from one sample cup and used the same batch of calibrators. tPSA and fPSA results were obtained in single determinations, BPHA and p2PSA in duplicate determination. The analysis was repeated in case of a coefficient of variation >20%.

Statistical analysis

All statistical analyses were performed using the SPSS v.15.0 software package (SPSS, Chicago, IL, USA). Differences between men with and without PCa in median levels of tPSA, fPSA, BPHA, and p2PSA were examined using the Mann-Whitney U test with two-sided p values. Calculated derivatives of the assayed variables included Hybritech phi, Beckman Coulter's mathematical combination of PSA, fPSA, and p2PSA. Beckman Coulter phi was calculated as (p2PSA/fPSA) x √(tPSA), representing a simplified version of a logistic regression model developed at Beckman Coulter. In addition, %fPSA (fPSA/tPSA x 100) and %p2PSA (p2PSA/fPSA x 100) were calculated. The areas under the receiver operator characteristic (ROC) area under the curve (AUC) as well as sensitivity and specificity were calculated to assess the diagnostic performances in PCa detection of the various assays. AUCs

of the ROC curves were compared using the method of DeLong *et al*[207]. In addition, p2PSA was added to a multivariate model based on tPSA and fPSA to specifically assess its additive value. Furthermore, using the Mann- Whitney U test with two-sided p values, median values of all assessed variables were compared in tumours with biopsy or pathologic Gleason score below or above 7. The number of missed cancers with biopsy or pathologic Gleason score of ≥7 was calculated at sensitivities of 95% or 90% for tPSA, %fPSA, %p2PSA, and phi.

Results

Patient characteristics are depicted in Table 1. Overall characteristics did not differ significantly except for the number of unknown pathologic Gleason scores and the number of men who underwent prostatectomy. In both cohorts, the percentage of tumours with a pathologic Gleason score ≥7 was higher than at the initial biopsy because of upgrading based on the prostatectomy specimen.

Table 1. Characteristics of both cohorts of men with and without prostate cancer to assess the additive value of p2PSA and BPHA next to total PSA and free PSA.

	Site 1	Site 2	P-value
Total no. of included men	405	351	
PCa cases (%)	226 (55.8)	174 (49.6)	ns
Non-PCa cases (%)	179 (44.2)	177 (50.4)	ns
Age range (yrs) (median)	55-75 (66)	50-77 (60)	ns
tPSA range (ng/ml) (median)	2.0-10.0 (4.4)	2.0-9.7 (4.3)	ns
Biopsy Gleason score (%)			
<7	168 (74.3)	122 (70.1)	ns
≥7	55 (24.3)	48 (27.6)	ns
Unknown	3 (1.3)	4 (2.3)	ns
No. of men that underwent prostatectomy (%)	162 (71.7)	174 (100.0)	p<0.0001
Pathological Gleason score (%)			
<7	96 (59.3)	88 (50.6)	ns
≥7	53 (32.7)	86 (49.4)	ns
Unknown	13 (8.0)	0 (0)	p=0.0001

At site 1, median levels of all assayed and calculated variables except BPHA differed significantly between men with and without PCa, with %fPSA, p2PSA, %p2PSA, and phi achieving the highest statistical significances (Table 2). At site 2, median levels of %fPSA, p2PSA, %p2PSA, and phi also differed significantly, in contrast with tPSA and fPSA, which did not differ between men with and without PCa. As BPHA was not significantly different between men with and without PCa in both cohorts, it was excluded from further analysis regarding PCa detection.

Table 3 shows the AUCs and specificities at sensitivities of 95% and 90% of the various univariate variables in both cohorts. In addition, Table 3 shows the specific additive effect of p2PSA to tPSA and fPSA by adding p2PSA to a multivariate logistic regression model consisting of tPSA and fPSA.

Table 2. Median levels of total PSA, free PSA, percent free PSA, BPHA, p2PSA, percent p2PSA, and Beckman Coulter Prostate Health Index in men with and without prostate cancer at sites 1 and 2 (ranges are depicted within brackets).

	Site 1			Site 2		
	PCa	non-PCa	P-value	PCa	non-PCa	P-value
tPSA (ng/ml)	4.69 (8.00)	4.34 (7.80)	0.003	4.44 (7.74)	4.03 (7.74)	0.3
fPSA (ng/ml)	0.65 (2.02)	0.76 (1.84)	0.004	0.48 (1.46)	0.49 (2.31)	0.5
%fPSA	14.07 (31.88)	18.4 (32.21)	<0.0001	11.16 (22.96)	12.63 (24.85)	0.01
p2PSA (ng/L)	10.90 (31.81)	9.31 (38.05)	<0.0001	8.01 (113.30)	6.20 (369.04)	<0.0001
%p2PSA	1.66 (5.92)	1.27 (2.87)	<0.0001	1.71 (21.61)	1.24 (31.06)	<0.0001
BPHA (ng/L)	157.30 (791.65)	180.52 (940.18)	0.1	137.50 (977.08)	135.84 (828.17)	0.7
phi	36.67 (108.93)	26.08 (73.01)	<0.0001	34.67 (416.24)	25.60 (910.29)	<0.0001

Table 3. Univariate and multivariate analyses to assess the predictive value in prostate cancer detection and specificity at 95% and 90% sensitivity. The multivariate model compares the cancer predictive values of total PSA and free PSA (= base model) with or without p2PSA (confidence intervals are depicted within brackets; * = compared to the AUC of phi).

Site 1				
	Specificity(%)			
Sensitivity	95% (95% CI)	90% (95% CI)	AUC (95% CI)	P-value*
Univariate:				
tPSA (ng/ml)	9.7 (5.7-15.0)	16.5 (11.3-22.8)	0.585 (0.535-0.634)	<0.001
fPSA (ng/ml)	6.0 (2.3-9.3)	15.6 (10.7-21.8)	0.582 (0.532-0.631)	<0.001
%fPSA	5.7 (2.8-10.2)	22.2 (16.3-29.0)	0.675 (0.627-0.721)	0.009
p2PSA (ng/L)	9.5 (5.6-14.8)	20.1 (14.5-26.7)	0.614 (0.565-0.662)	<0.001
%p2PSA	20.1 (14.5-26.7)	31.8 (25.1-39.2)	0.716 (0.669-0.759)	0.03
phi	22.7 (16.8-29.6)	34.7 (27.7-42.2)	0.750 (0.704-0.791)	-
Multivariate:				
Base model	8.0 (4.4-13.0)	21.6 (15.8-28.4)	0.675 (0.627-0.720)	0.006
Base model + p2PSA	23.9 (17.8-30.9)	30.1 (23.4-37.5)	0.755 (0.710-0.796)	0.5
Site 2				
	Specificity (%)			
Sensitivity	95% (95% CI)	90% (95% CI)	AUC (95% CI)	P-value*
Univariate:				
tPSA (ng/ml)	7.9 (4.4-12.9)	10.2 (6.1-15.6)	0.534 (0.473-0.594)	<0.001
fPSA (ng/ml)	9.0 (5.3-14.3)	11.3 (7.0-16.9)	0.521 (0.467-0.577)	<0.001
%fPSA	9.0 (5.3-14.3)	11.9 (7.5-17.6)	0.576 (0.523-0.629)	<0.001
p2PSA (ng/L)	12.4 (8.0-18.2)	19.2 (13.7-25.8)	0.625 (0.572-0.675)	<0.001
%p2PSA	21.5 (15.7-28.3)	33.9 (27.0-41.4)	0.695 (0.644-0.743)	0.3
phi	30.5 (23.8-37.9)	31.1 (24.3-38.5)	0.709 (0.658-0.756)	-
Multivariate:				
Base model	7.3 (4-0-12.2)	12.4 (8.0-18.2)	0.581 (0.527-0.633)*	0.001
Base model + p2PSA	23.2 (17.2-30.1)	36.2 (29.1-43.7)	0.697 (0.646-0.744)	0.3

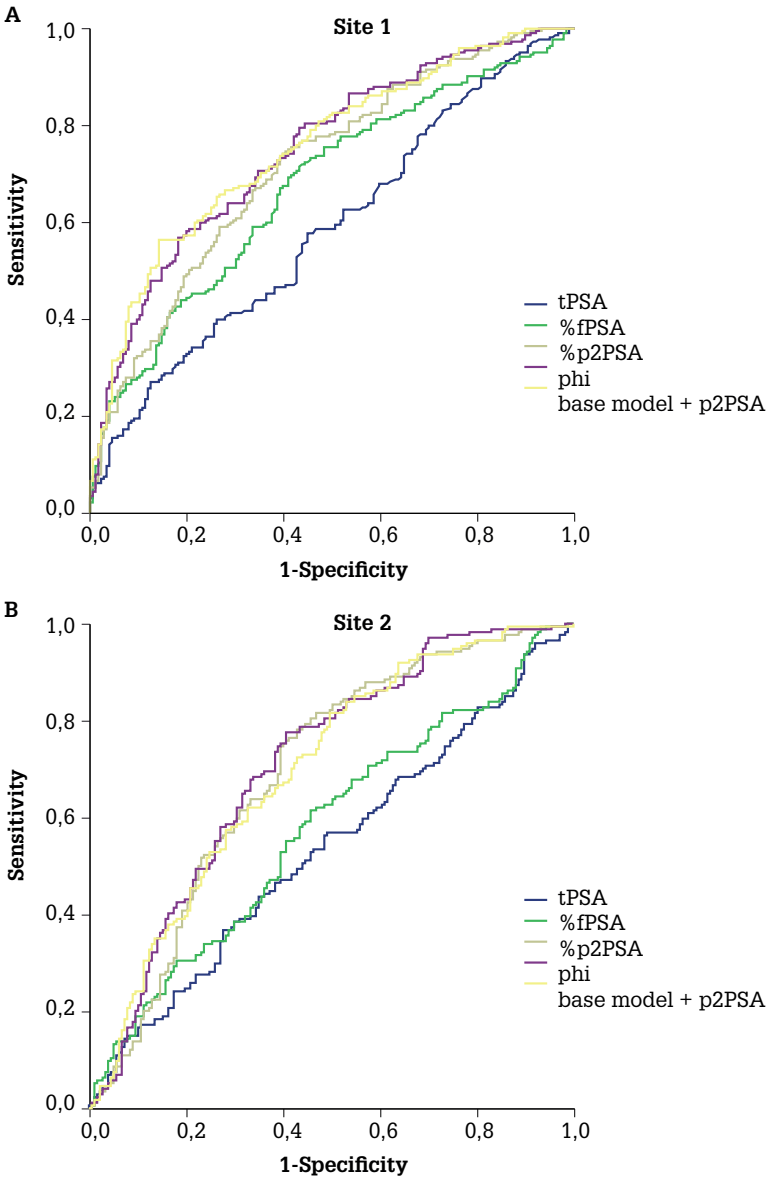

Figure 1. Receiver operator characteristic curves of total PSA, percent free PSA, percent p2PSA, Beckman Coulter Prostate Health Index, and the base model with p2PSA at (a) site 1 and (b) site 2 (tPSA = total prostate-specific antigen; %fPSA = percent free prostate specific antigen; %p2PSA = percent p2PSA; phi = Beckman Coulter Prostate Health Index).

Table 4. Median values of total PSA, free PSA, p2PSA, and derivatives in serum of patients from site 1 and site 2 with tumours having biopsy or pathologic Gleason scores less or more than 7.

Site 1						
	Biopsy GS <7	Biopsy GS ≥7	P-value	Pathological GS <7	Pathological GS ≥7	P-value
Univariate:						
tPSA (ng/ml)	4.55	5.58	0.06	4.74	5.58	0.1
fPSA (ng/ml)	0.68	0.60	0.03	0.66	0.64	0.4
p2PSA (ng/L)	10.86	11.17	0.9	11.09	11.08	0.9
BPHA (ng/L)	150.60	174.54	0.6	149.21	204.24	0.3
%fPSA	15.54	11.42	<0.0001	14.51	11.9	0.01
%p2PSA	1.60	1.91	0.002	1.63	1.84	0.09
phi	34.20	43.03	<0.0001	36.32	42.38	0.02
Multivariate:						
Base model	1.29	1.76	<0.0001	1.41	1.68	0.02
Base model + p2PSA	1.53	2.19	<0.0001	1.69	2.19	0.008
Site 2						
	Biopsy GS <7	Biopsy GS ≥7	P-value	Pathological GS <7	Pathological GS ≥7	P-value
Univariate:						
tPSA (ng/ml)	4.13	5.37	0.004	4.34	4.80	0.3
fPSA (ng/ml)	0.45	0.54	0.03	0.48	0.47	0.8
p2PSA (ng/L)	7.86	8.56	0.1	8.87	7.46	0.1
BPHA (ng/L)	123.59	210.84	0.001	130.08	144.33	0.4
%fPSA	11.16	11.01	0.7	11.50	10.88	0.1
%p2PSA	1.75	1.57	0.6	1.84	1.60	0.3
phi	34.05	35.83	0.2	35.51	33.95	0.5
Multivariate:						
Base model	0.98	1.04	0.3	0.96	1.05	0.1
Base model + p2PSA	1.11	1.15	0.3	1.11	1.14	0.8

To investigate the influence of DRE results, all analyses described above were repeated after exclusion of all men with suspicious DRE results. This produced nearly identical results (data not shown). To assess the relationship between the assayed variables and Gleason score, median values were related to biopsy and pathologic Gleason score above or below 7. At site 1, the highest statistical difference in relationship to biopsy Gleason score was achieved by %fPSA, phi, and the base model with or without p2PSA (Table 4).

In relationship to pathologic Gleason score, the base model including p2PSA achieved the highest clinical significance followed by %fPSA and phi. In contrast, at site 2, the highest statistical difference in relationship to biopsy Gleason score was reached by BPHA followed by tPSA and fPSA. None of the assayed variables reached statistical significance in discriminating tumours with pathologic Gleason score above or below 7. In addition, we assessed which variable would miss the least number of tumours with a biopsy or pathologic Gleason score ≥7. At site 1, at 95% and 90% sensitivity, %p2PSA, phi, and the base model with or without p2PSA missed the least number of tumours with a biopsy or pathologic Gleason score ≥7 (Table 5). At site 2, at 95% sensitivity, %p2PSA and the base model consisting of fPSA and tPSA missed the least number of tumours with a biopsy Gleason score of ≥7. At 90% sensitivity, the base model with the addition of p2PSA missed the least number of tumours with a biopsy Gleason score ≥7. At 95% sensitivity, %p2PSA missed the least number of tumours with a pathologic Gleason score ≥7, whereas at 90% sensitivity, the least number of tumours with a pathologic Gleason score ≥7 were missed by the base model with p2PSA included.

Table 5. Cancers with biopsy or pathologic Gleason scores ≥7 missed by total PSA, percent free PSA, percent p2PSA, Beckman Coulter Prostate Health Index, and multivariate models at 95% and 90% sensitivity at both sites.

Site 1	Cut-off		No. biopsy GS ≥7				No. pathological GS ≥7			
			Missed		Detected		Missed		Detected	
Sensitivity	95%	90%	95%	90%	95%	90%	95%	90%	95%	90%
Univariate:										
tPSA (ng/ml)	2.56	2.93	4	6	51	49	3	5	50	48
%fPSA	28.10	22.95	1	2	54	53	1	2	52	51
%p2PSA	0.91	1.05	1	1	54	54	1	2	52	51
phi	19.43	22.49	1	1	54	54	1	1	52	52
Multivariate:										
Base model	0.30	0.62	1	1	54	54	1	1	52	52
Base model + p2PSA	0.18	0.39	1	1	54	54	1	1	52	52
Site 2	Cut-off		No. biopsy GS ≥7				No. pathological GS ≥7			
			Missed		Detected		Missed		Detected	
Sensitivity	95%	90%	95%	90%	95%	90%	95%	90%	95%	90%
Univariate:										
tPSA (ng/ml)	2.36	2.50	1	3	47	45	7	10	79	76
%fPSA	17.65	19.12	2	4	46	44	4	7	82	79
%p2PSA	0.89	1.05	0	4	48	44	1	8	85	78
phi	20.45	20.89	2	2	46	46	3	6	83	80
Multivariate:										
Base model	0.56	0.64	0	4	48	44	2	7	84	79
Base model + p2PSA	0.41	0.55	1	1	47	47	2	5	84	81

Discussion

The present two-centre study was initiated to assess the performance in PCa detection of the PSA isoforms p2PSA and BPHA in addition to tPSA and fPSA. Furthermore, the relationship between p2PSA and BPHA as well as biopsy and pathologic Gleason score and their ability to specifically detect cancers with a Gleason score ≥7 was assessed. In short, BPHA had no additional PCa predictive value over tPSA and %fPSA, whereas phi and %p2PSA showed significantly higher PCa predictive values combined with an increased specificity at 95% and 90% sensitivity (Table 3). This pattern was observed in both patient cohorts. Furthermore,

multivariate analysis showed that the addition of p2PSA to a logistic regression model consisting of tPSA and fPSA significantly increased PCa predictive value and specificity (Table 3). Exclusion of men with suspicious DRE results resulted in similar outcomes (data not shown). Regarding BPHA, our findings are similar compared to those of prior reports, which showed that BPHA as a single marker is unable to predict the presence of PCa[59,60,63,82]. However, BPHA might be of limited additional value in combination with existing markers, as was illustrated by a recent study by Stephan *et al.*, but these results could not be reproduced by the present study (data not shown)[204]. In contrast, p2PSA and %p2PSA levels have been shown to be significantly elevated in men with PCa, resulting in an increase in PCa predictive value compared to %fPSA[61,62,202,203]. These results are confirmed in the present study, not only by univariate analysis but also by the addition of p2PSA to a logistic regression model consisting of tPSA and fPSA (Tables 2 and 3). In addition, the present study evaluated the performance of phi, a calculated derivative of p2PSA, fPSA, and tPSA. Comparable to p2PSA levels, phi is significantly elevated in men with PCa (Table 2). With respect to PCa predictive value, phi performed significantly better than tPSA and %fPSA. In cohort 1, phi performed significantly better than %p2PSA, whereas in cohort 2, no difference was observed. When compared to the multivariate model consisting of tPSA, fPSA, and p2PSA, no significant difference in PCa predictive value was observed. Unfortunately, as data regarding prostate volume were lacking in a large proportion of patients, the performance of tPSA density and other density derivatives of the assayed variables could not be assessed. Regarding the detection of PCa with a Gleason score ≥7, phi, %fPSA, and the multivariate model with or without p2PSA performed equally in cohort 1, so showing no specific additional value of p2PSA (Tables 4 and 5). At site 2, BPHA and tPSA showed the highest significance in relationship to biopsy Gleason scores, whereas none of the assayed variables showed a significant relationship with pathologic Gleason score. Overall, no large differences between the various variables regarding the specific detection of PCa with Gleason score ≥7 were observed. These results are in contrast with Catalona *et al.*[81] and Stephan *et al.*[203], who showed that %p2PSA had selectivity for detecting PCa with Gleason score ≥7. Another observation is the fact that tPSA and fPSA concentrations did not differ significantly between men with and without PCa at site 2 in contrast with site 1. These differences may largely result from discrepancies in the assays and nomograms used for initial patient screening, resulting in cohorts with different PCa characteristics (see section 2). A second explanation could be that at site 2, a greater proportion of PCa cases were detected in later screening rounds. It has been shown that the predictive value of tPSA decreases in successive screening rounds[208-210]. This might be reflected in the fact that no significant relationship between tPSA

and the presence of PCa was observed in cohort 2. Furthermore, the observed differences between the two cohorts regarding biopsy and pathologic Gleason score and the assayed variables, especially BPHA, might be contributable to the fact that Gleason scores were assigned by different pathologists at both centres. In addition, the number of patients with a known pathologic Gleason score might have been too small to detect significant relationships. Another point of concern could be the risk of a type one error resulting from the number of statistical tests and using an α level of 0.05. However, to reduce the risk of a type two error, we have chosen not to apply a correction method, such as the Bonferroni correction. Although differences between both PCa cohorts exist, p2PSA and phi differ significantly between men with and without PCa in both cohorts and are able to correctly predict the presence of PCa. In addition, p2PSA showed a specific additional increase in PCa predictive value when added to a multivariate model consisting of tPSA and fPSA. p2PSA has limited additional value in identifying men with aggressive PCa (Gleason score ≥7). Further study is needed to clearly define the definitive value of p2PSA in PCa detection and its ability to modify the number of men undergoing prostate biopsy. Especially, the performance of phi should be further evaluated, as other data investigating phi are currently lacking. Preferentially, these should be prospective studies based on direct PCa screening by p2PSA.

Conclusions

The present study shows that p2PSA and, moreover, %p2PSA and phi could have additional value with respect to tPSA and %fPSA in PCa detection within the tPSA range of 2– 10 ng/ml by significantly increasing the predictive value and specificity for PCa. By increasing the clinical specificity of phi and p2PSA relative to tPSA and fPSA, the use of phi and p2PSA could potentially modify the number of men receiving a recommendation for biopsy. In addition, p2PSA has limited additional value in identifying men with aggressive PCa (Gleason score ≥7).

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CHAPTER 5

CLINICAL IMPACT OF NEW PROSTATE-SPECIFIC ANTIGEN WHO STANDARDISATION ON BIOPSY RATES AND CANCER DETECTION

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Abstract

Background: Clinicians may be unaware that replacement of the historical total prostate-specific antigen (tPSA) standard with the WHO 96/670 international standard leads to difficulties in interpreting tPSA results. Our aim was to investigate the relationship between the Hybritech and WHO calibrations of the Beckman Coulter tPSA assay, and to assess the impact on prostate cancer (PCa) detection.
Methods: tPSA concentrations were measured in 106 serum samples with both Hybritech and WHO calibrations. The established relationships were used for an in silico experiment with a cohort of 5865 men. Differences in prostate biopsy rates, PCa detection, and characteristics of missed cancers were calculated at biopsy thresholds of 3.0 and 4.0 ng/ml.
Results: A linear relationship was observed between the 2 calibrations, with a 20.3% decrease in tPSA values with the WHO standard compared with the Hybritech calibration. Applying the WHO calibration to the cohort of 5865 men yielded a 20% or 19% decrease in prostate biopsies and a 19% or 20% decrease in detected cancers compared with the Hybritech calibration, at a cut-off for biopsy of 3.0 or 4.0 ng/ml, respectively. The decrease in detected cancers declined to 9% or 11% if an abnormal result in a digital rectal examination or a transrectal ultrasound evaluation was used as trigger for prostate biopsy (cut-off of 3.0 or 4.0 ng/ml, respectively).
Conclusions: Application of the WHO standard for tPSA assays with commonly used tPSA thresholds leads to a significant decrease in PCa detection. Careful assessment of the relationship between the WHO standard and the thresholds used for prostate biopsy is hence necessary.

Introduction

Numerous prostate-specific antigen (PSA) reagent sets are available for the measurement of total PSA (tPSA) concentrations. Several publications have shown that this wide variety of assays leads to very different values for tPSA. These findings cannot always be satisfactorily explained, but possible underlying reasons include differences in assay design, such as differential epitope recognition, cross-reactivity with PSA-homologous antigens, and the lack of equimolarity[5,112-115]. The last issue, which causes tPSA assays to overreport tPSA concentrations in patients with benign prostate hyperplasia compared with those with prostate cancer (PCa), has largely been resolved through major modifications in many of the tPSA assays. Several studies have reported a small improvement in diagnostic performance, which has been attributed to changes in the equimolar response[211,212]. If the assay method applied and the specific reference range for the assay are unknown to the

clinician, tPSA values may be misinterpreted, possibly influencing the decision to recommend that patients undergo a prostate biopsy for PCa detection[113]. There may be clinical implications if serial tPSA measurements are used to calculate tPSA velocity or doubling time for the purpose of indicating PCa aggressiveness or treatment failure[110,213-218]. In addition, the lack of interchangeability of the various assays may prevent objective judgment and unbiased comparison of the results of previous studies. The First Stanford Conference was convened in 1992 to improve the interchangeability of tPSA assays. The Second Stanford Conference resulted in a reference standard consisting of 90% purified PSA– antichymotrypsin and 10% free PSA on a molar basis[120]. The WHO adoption of this standard resulted in the First International Standard for tPSA under the National Institute of Biological Standards and Control, also known as WHO 96/670[116,117]. Later, the WHO also introduced a standard for free PSA[116,117]. Differences among tPSA assays seem to have decreased since the introduction of the WHO 96/670 calibration, but they have not been completely resolved and eliminated[118-120]. Therefore, the term "harmonization" has become favoured instead of "standardisation," because not all assays perform equally, even after standardisation[219,220]. To minimize the likelihood of clinical errors, several countries now require their laboratories to report tPSA concentrations in accordance with the WHO 96/670 preparation[221]. Although most clinicians are familiar with the fact that different tPSA assays produce different results, they may not be aware that restandardisation from a historical standard to the WHO calibration causes a shift in mass units, which yields a potential for underestimating tPSA values. If this shift is not fully appreciated, especially with respect to the tPSA threshold value for biopsy, a resulting decrease in PCa detection may produce life-altering consequences for individual patients. Thus, different cut-off values are required for the historical standard and the WHO calibration to achieve the same clinical performance. With the introduction of the WHO 96/670 calibration for tPSA, however, the question has arisen as to how this calibration would influence the clinical outcomes for the current tPSA thresholds. It is clear that the thresholds and methods used to measure tPSA are coupled; therefore, maintaining the established clinical validity requires that significant changes in the assay be evaluated for changes in reference values and cut-off thresholds. We describe our study focusing on the effects of implementing the WHO standard on the Beckman Coulter tPSA assay. This assay, the first tPSA assay to be approved by the US Food and Drug Administration, established the conventional 4.0- ng/ml cut-off value for prostate biopsy on the basis of a large multicenter prospective study by applying the gold standard Hybritech tPSA assay[222]. Although age-specific reference intervals are now available for tPSA, the 4.0- ng/ml tPSA threshold for prostate biopsy is well known, not only by healthcare professionals but also by the general public. We also applied a

cut-off value of 3.0- ng/ml because this concentration is currently used as indication for prostate biopsy by the European Randomized Study of Screening for Prostate Cancer (ERSPC)[223].

Methods

An overview of the analyses and an outline of the present study are depicted in Figure 1. First, we used the Access Hybritech Total PSA assay with both the Hybritech calibrator and the WHO calibrator to measure tPSA concentrations in 106 fresh serum samples to compare the Beckman Coulter Hybritech calibration with the WHO 96/670 standard.

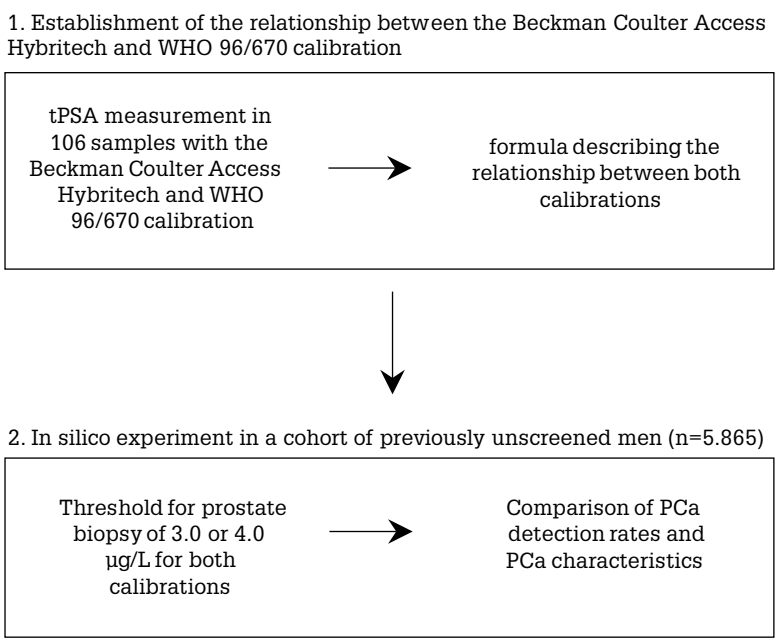

Figure 1. Overview of the present study. After establishing the relationship between the WHO 96/670 and Hybritech calibrations, we performed an in silico experiment to assess the clinical implications of the WHO 96/670 reference preparation.

The 106 serum samples were obtained from men included in the screening program of the Rotterdam center of the ERSPC. The study was approved by the Medical Ethics Committee of the Erasmus MC[224]. Hybritech tPSA concentrations varied from

0.1 ng/ml to 9.1 ng/ml. PCa was detected after prostate biopsy in 4 of the 106 patients. The relationship between the WHO 96/670 and Hybritech tPSA calibrations was calculated with the Cusum test for linearity[225]. The resulting formula was then used to perform an in silico calculation of the tPSA concentrations based on the WHO 96/670 standard for a cohort of men selected from the database of the Rotterdam center of the ERSPC. Patients were eligible for inclusion if they had undergone sextant biopsy of the prostate. Men were biopsied in the study either because of a Hybritech tPSA concentration ≥3.0 ng/ml or because of a Hybritech tPSA concentration of <3.0 ng/ml at a repeat screening during a side study to test the value of free PSA for PCa detection[226,227]. Digital rectal examination (DRE) and transrectal ultrasound (TRUS) results were known for all men. All of the 5865 men included in the study were unbiopsied at the start of the study and were 55 to 75 years of age. The median tPSA concentration was 4.0 ng/ml (range, 0.1–315.7 ng/ml; see Figure 2. On the basis of the sextant biopsy results, PCa was diagnosed in 1539 patients (26%) in this cohort, 539 of whom eventually underwent a radical prostatectomy (RP). The characteristics of the PCa patients within the cohort are summarized in Table 1. This cohort of 5865 men was used in an in silico experiment that evaluated cut-off values for prostate biopsy of ≥3.0 ng/ml and ≥4.0 ng/ml for both the Hybritech and WHO 96/670 calibrations. We subsequently calculated differences in prostate biopsy rates, PCa detection, and characteristics of the missed cancers. The SPSS 12.0 software package was used for statistical analyses.

Table 1. Characteristics of PCa patients in a cohort of previously unbiopsied men from the Rotterdam center of the ERSPC (n=1539).

		Frequency
Clinical stage	T1	727 (47%)
	T2	607 (39%)
	T3	139 (13%)
	T4	12 (1%)
Biopsy Gleason score	<7	1059 (69%)
	7	375 (24%)
	>7	96 (6%)
	unknown	9 (1%)
Gleason score of RP specimen (n=539)	<7	323 (60%)
	7	183 (34%)
	>7	27 (5%)
	unknown	6 (1%)

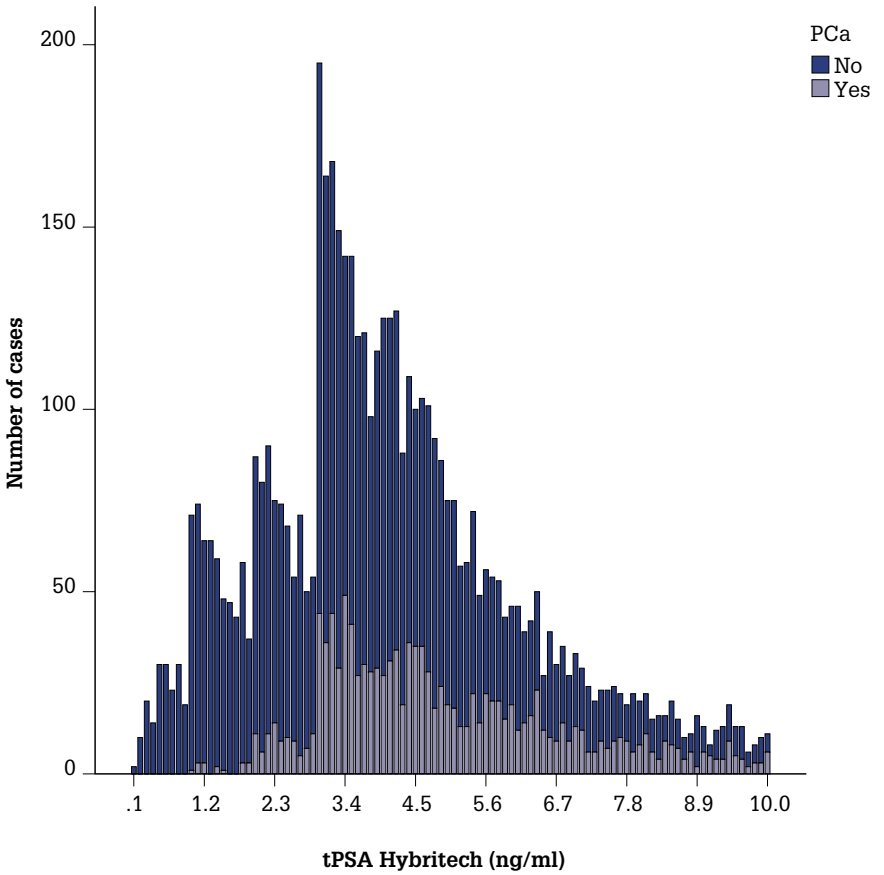

Figure 2. Distribution of total PSA values in PCa and non-PCa cases in the cohort of previously unbiopsied men between tPSA 2-10 ng/ml.

Results

We observed a linear relationship between the Hybritech and WHO 96/670 tPSA calibrations. This relationship is described by the formula: WHO calibration = (0.7964 x Hybritech calibration) + 0.007 ng/ml. The 95% confidence intervals for the slope and the y intercept were 0.78–0.81 and -0.004–0.024 ng/ml, respectively (Figure 3).

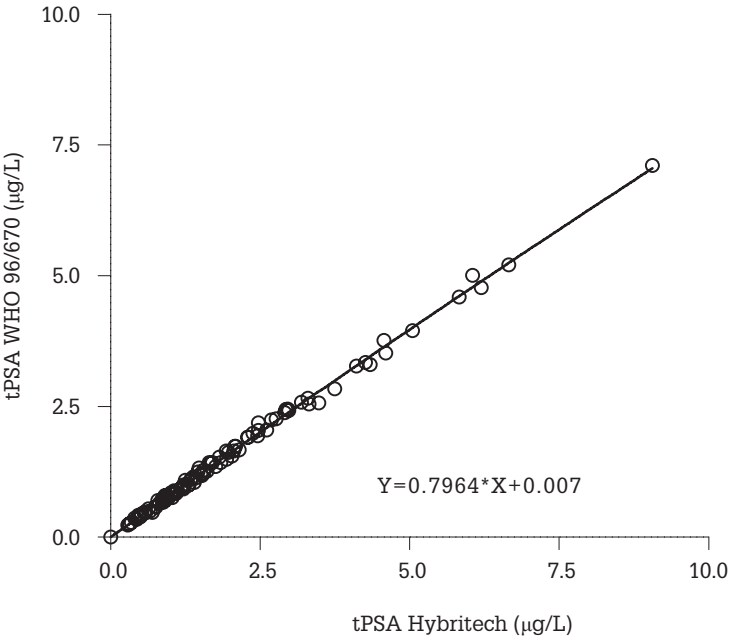

Figure 3. tPSA concentrations measured with the Beckman Coulter Hybritech calibration plotted against those obtained with the WHO 96/670 calibration (n=106).

Use of this formula to calculate the WHO 96/670 tPSA concentrations for the cohort of previously unbiopsied men (characteristics summarized in Table 1) yielded a 20.3% decrease in tPSA concentrations compared with the known Hybritech tPSA concentrations. A tPSA biopsy threshold of ≥3.0 ng/ml for the Hybritech calibration yielded 4419 biopsied patients from the 5865 cases (75%), leading to a total of 1430 detected PCa cases (92%) (Table 2). A tPSA biopsy threshold of ≥4.0 ng/ml for the Hybritech calibration yielded 3004 biopsied patients (51%), leading to a total of 1073 detected PCa cases (70%). Without adjusting the cut-offs for the WHO 96/670 tPSA calibration (i.e., ≥3.0 or ≥4.0 ng/ml, which is equal to a Hybritech tPSA cut-off of ≥3.6 or ≥4.8 ng/ml, respectively), one obtains 3218 men (55%) as candidates for prostate biopsy, leading to 1130 detected cases of PCa (73%) with a cut-off of ≥3.0 ng/ml (Table 2).

Table 2. In silico analysis of the clinical implications of the new PSA WHO calibration.

	PSA Hybritech calibration (biopsy threshold ≥3 ng/ml)	PSA WHO 96/670 calibration (biopsy threshold ≥3 ng/ml)
No. of biopsied patients (% of all men in cohort)	4,419 (75%)	3,218 (55%)
No. of detected PCa cases (% of all PCa cases in cohort)	1,430 (92%)	1,130 (73%)
	PSA Hybritech calibration (biopsy threshold ≥4 ng/ml)	PSA WHO 96/670 calibration (biopsy threshold ≥4 ng/ml)
No. of biopsied patients (% of all men in cohort)	3,004 (51%)	1,873 (32%)
No. of detected PCa cases (% of all PCa cases in cohort)	1,073 (70%)	767 (50%)

Nonadjustment yields a 20% decrease in the number of prostate biopsies (from 75% to 55%) and a 19% decrease tPSA biopsy threshold of ≥3.0 ng/ml for the WHO 96/670 calibration would miss 300 PCa cases that would have been detected with the Hybritech tPSA calibrator at the same threshold for prostate biopsy. At a cut-off of ≥4.0 ng/ml for the WHO 96/670 calibration, 1873 men (32%) were candidates for prostate biopsy, leading to 767 detected cases of PCa (50%) and yielding a 19% decline in the biopsy rate and a subsequent 20% decrease in PCa detection. In the group of patients who underwent an RP, 507 (94%) of 539 cancers were detected by applying the Hybritech tPSA calibration at a biopsy threshold of ≥3.0 ng/ml, in contrast with the WHO calibration detecting 406 (75%) of 539 PCa cases, a decrease of 19%. Thus, use of the WHO calibration with a tPSA biopsy threshold of ≥3.0 ng/ml specifically missed 101 RP cases that would have been detected with the Hybritech tPSA calibrator at the same threshold for prostate biopsy. At a cut-off of ≥4.0 ng/ml, application of the Hybritech tPSA calibration detected 386 (72%) of 539 cancers, in contrast with 272 (50%) of 539 PCa cases detected with the WHO calibration. The clinical and pathologic stages and the biopsy and RP sample Gleason scores of the missed cancers are depicted in Tables 3 and 4. At biopsy thresholds of ≥3.0 ng/ml and ≥4.0 ng/ml, there are significant differences in cancer characteristics between the cancers that are missed by application of the WHO 96/670 standard and the cancers that are detected with the Hybritech calibration. Overall, the group of missed cancers consists of tumours with lower clinical and pathologic tumour stages and lower biopsy and RP Gleason scores. At a 3.0- ng/ml cut-off, an abnormal DRE result is found in 109 (36%) of the 300 missed cancer cases, and an abnormal TRUS result is present in 97 (32%) of the 300 missed cancer cases. When an abnormal DRE or TRUS result is used as a trigger for prostate biopsy, 146 (49%)

of the 300 missed cancer cases will be detected despite the application of the WHO 96/670 reference standard. This approach yields a decrease in PCa detection of 9% compared with the Hybritech calibration. At a threshold of 4.0 ng/ml, an abnormal DRE result and an abnormal TRUS result are found in 111 cases (36%) and 90 cases (29%), respectively, of the 306 missed PCa cases. Use of an abnormal DRE or TRUS result as a trigger for prostate biopsy results in the detection of 139 (45%) of the 306 missed cancer cases, leading to a decrease in PCa detection of 11% compared with the Hybritech calibration. Biopsy frequencies and PCa-detection rates were similar after the linear relationship between the 2 calibrations was used to correct the biopsy threshold for the WHO 96/670 calibration to 2.4 or 3.2 ng/ml.

Table 3. Characteristics of PCa cases detected by the Hybritech calibration (column 2) and of PCa cases specifically missed by applying the WHO 96/670 calibration that were detected by the Hybritech calibration (column 3), at tPSA biopsy thresholds of ≥3.0 ng/ml and ≥4.0 ng/ml.

Biopsy threshold of ⩾ 3.0 ng/ml		PCa cases detected by Hybritech calibration (n=1430)	PCa cases specifically missed by the WHO 96/670 calibration (n=300)	P-value
Clinical stages	T1	647 (45%)	154 (51%)	ns
	T2	583 (41%)	130 (43%)	ns
	T3	188 (13%)	15 (5%)	<0.001
	T4	12 (1%)	1 (0%)	ns
Biopsy Gleason score	<7	966 (68%)	241 (80%)	<0.001
	7	361 (25%)	49 (16%)	<0.001
	>7	94 (7%)	10 (3%)	0.03
	unknown	9 (1%)	1 (0%)	ns
Biopsy threshold of ⩾ 4.0 ng/ml		**PCa cases detected by Hybritech calibration (n=1073)**	**PCa cases specifically missed by the WHO 96/670 calibration (n=306)**	**P-value**
Clinical stages	T1	468 (44%)	166 (54%)	<0.001
	T2	427 (40%)	111 (36%)	ns
	T3	167 (16%)	29 (10%)	0.007
	T4	11 (1%)	0 (0%)	ns
Biopsy Gleason score	<7	677 (63%)	236 (77%)	<0.001
	7	304 (28%)	59 (19%)	0.002
	>7	83 (8%)	11 (4%)	0.01
	unknown	9 (1%)	0 (0%)	ns

Table 4. Characteristics of RP cases that were detected with the Hybritech calibration (column 2) and of RP cases specifically missed by applying the WHO 96/670 calibration that were detected with the Hybritech calibration (column 3), at biopsy thresholds of ≥3.0 ng/ml and ≥4.0 ng/ml.

Biopsy threshold of ≥3.0 ng/ml		PCa cases detected by Hybritech calibration n(=507)	RP cases specifically missed by the WHO 96/670 calibration (n=101)	P-value
Pathological stages	T2	387 (76%)	88 (87%)	0.02
	T3	98 (19%)	11 (11%)	0.04
	T4	21 (4%)	2 (2%)	ns
	unknown	1 (0%)	0 (0%)	ns
Gleason score of RP specimen	<7	300 (59%)	65 (64%)	ns
	7	175 (35%)	33 (33%)	ns
	>7	26 (5%)	3 (3%)	ns
	unknown	6 (1%)	0 (0%)	ns
Biopsy threshold of ≥4.0 ng/ml		PCa cases detected by Hybritech calibration (n=386)	RP cases specifically missed by the WHO 96/670 calibration (n=114)	P-value
Pathological stages	T2	283 (73%)	97 (85%)	0.01
	T3	83 (22%)	13 (11%)	0.02
	T4	19 (5%)	4 (4%)	ns
	unknown	1 (0%)	0 (0%)	ns
Gleason score of RP specimen	<7	223 (58%)	77 (68%)	ns
	7	134 (35%)	30 (26%)	ns
	>7	23 (6%)	6 (5%)	ns
	unknown	6 (2%)	1 (1%)	ns

Discussion

Our results show that application of the WHO 96/670 calibration for tPSA yields a 20.3% decrease in tPSA values compared with the historical Beckman Coulter Hybritech calibration. If the threshold for prostate biopsy is not adjusted accordingly, there will be a significant decline in prostate biopsy rates (20% or 19%) and a subsequent decrease in detected PCa cases (19% or 20%) for the Beckman Coulter tPSA assay at commonly used cut-offs for biopsy (3.0 and 4.0 ng/ml, respectively). This decrease in detected PCa cases is limited to 9% or 11% if an abnormal DRE or TRUS result is used as a trigger for prostate biopsy (threshold of 3.0 or 4.0 ng/ml, respectively). In addition, significant shifts in tumour stages and Gleason scores were observed with the application of the WHO 96/670 tPSA calibration. Missed PCa cases showed fewer high-stage tumours and fewer tumours with high Gleason scores than for PCa cases that were detected with the Hybritech calibration at a biopsy threshold of ≥3.0 ng/ml or ≥4.0 ng/ml. Biopsy and PCa-detection rates were similar after the linear relationship between the WHO and Hybritech calibrations was used to correct the biopsy threshold for the WHO 96/670 calibration to 2.4 ng/ml or 3.2 ng/ml, respectively. One possible limitation of our study is that the second part is based on in silico calculations of WHO 96/670 tPSA concentrations, instead of on direct measurements of serum concentrations. This in silico calculation was based on the linear relationship established in a study of a smaller cohort (n=106) that used both calibrations for actual serum measurements; however, the 20.3% negative shift in tPSA values that is introduced by application of the WHO calibration is virtually identical to the findings of earlier studies by Kort *et al.* and Roddam *et al.*, who found a 22% and 19% difference, respectively, in tPSA concentrations (mean, 20.5%) between the WHO 96/670 and Hybritech calibrations[119,221]. Whereas we used an in silico experiment to evaluate the clinical effects of applying the WHO standard for tPSA, the actual relationship between the WHO calibration and the original Hybritech calibration was established with clinical serum samples. Because tPSA assays are known to be highly sensitive to the matrix of the sample, the use of clinical samples avoids biases that arise from the use of artificial samples[219]. A second limitation is that a cohort of screen detected PCas in asymptomatic men was used instead of a cohort of clinically detected PCa cases to assess the clinical impact of the new WHO calibration. Implications for a cohort of clinically detected PCas will be similar, more or less, with probably fewer missed PCa cases because of initially higher tPSA concentrations. We did not investigate the relationships between other tPSA assays and the new WHO standard in this study. This choice was partly because tPSA measurements made within the screening program of the ERSPC have always been based on the Hybritech system. The strategy has been to use the same method

during the entire study to minimize errors in medical decision making. Such errors may occur if different assays are used during patient monitoring. Kort *et al.* recently concluded that the results of other assays also deviate from expected tPSA values if the WHO 96/670 reference preparation is applied (regression slopes between 0.99 and 1.08), showing that further efforts are needed to improve the interchangeability of tPSA assays[118,119,221]. In addition, we emphasize that although use of a biopsy cut-off of 3.0 or 4.0 ng/ml is common practice, tPSA is a continuous, not a dichotomous, biomarker; therefore, the risk of PCa increases as the PSA concentration increases[228]. As far as we know, this study is the first to evaluate the clinical effects of the WHO 96/670 calibration for tPSA in a large screening cohort. Therefore, we have not been able to compare the number of missed cancers and the shifts in PCa characteristics with those of previous studies. Significant changes in PCa detection and PCa characteristics have also been observed in studies that have assessed the interchangeability of various tPSA assays, although a study by Yurdakul *et al.* concluded that different tPSA assays detected PCa cases with identical histological features[114,218,229,230]. This study and others, however, evaluated the effects of different tPSA assays, which have different performance characteristics. This approach contrasts with that of our study, in which the only difference was the reference standard. For the present study, the difference in cancer characteristics between the cancers missed by the WHO 96/670 calibration and the cancers detected by the Hybritech calibration can be explained by the distribution of tumour stages and Gleason scores in the entire population of the screening cohort. The group of missed cancers has Hybritech tPSA values of 3.0–3.6 ng/ml or 4.0–4.8 ng/ml and contains fewer high-stage and high-grade tumours than the cohort of all PCa cases with Hybritech tPSA concentrations ≥3.0 ng/ml or ≥4.0 ng/ml, respectively. The finding of a decrease in cancer detection caused by application of the WHO 96/670 calibration may also have positive implications in light of the current discussions on limiting PCa overdetection and subsequent overtreatment[231-234]. Because the group of missed cancers contains significantly fewer tumours with a high stage or a high Gleason score and more tumours with favourable characteristics (Tables 3 and 4), missing these cancers may not have clinical consequences and so may limit the overdetection and overtreatment of PCa. Furthermore, we have shown that the decrease in detected PCa cases caused by application of the WHO 96/670 standard is less dramatic when abnormal DRE or TRUS results are applied as triggers for prostate biopsy. Given the limited predictive value of an abnormal DRE or TRUS result in a screening setting, however, especially in the low tPSA range of approximately 3.0 ng/ml or 4.0 ng/ml, the actual decrease in PCa detection most likely will be closer to 19% or 20%, respectively, than to 9% or 11%[235-239]. The recalibration is also expected to affect PCa detection through effects on other PSA threshold values for biopsy.

Adaptation to the WHO 96/670 reference standard will also affect tPSA-derived variables, such as tPSA velocity, tPSA doubling time, and age-adjusted tPSA values, with potential consequences for the individual patient. In conclusion, the present study has shown that a decision to adapt tPSA testing to the WHO 96/670 reference standard requires both careful assessment of the relationship between the historical standard and the WHO standard and subsequent establishment of a new threshold for prostate biopsy. In this study, we found that the threshold for prostate biopsy shifted from 3.0 ng/ml or 4.0 ng/ml with the historical Hybritech calibration to 2.4 ng/ml or 3.2 ng/ml, respectively, after application of the WHO 96/670 calibration. If not adjusted, this leads to a 19% or 20% decrease in PCa detection. Hence, if clinicians are unaware of the replacement of their historical tPSA standard with the WHO 96/670 reference preparation and do not adapt the threshold concentration for prostate biopsy, the number of prostate biopsies and subsequent PCa-detection rates will shift considerably in their clinic.

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CHAPTER 6

GENERAL DISCUSSION

The xenograft model system detects novel PCa markers

In spite of major investments, decades of intensive research, and hundreds of papers proposing possible novel markers for PCa, only very few markers have reached the clinic. This is especially remarkable in the light of all the advanced techniques that are currently available to discover and validate possible novel markers, which did not exist during the time that PSA was discovered. A large portion of these failures is formed by so-called 'false discoveries', novel markers that seem initially highly promising in the discovery phase but fail to show clinical significance in the validation phase[240]. An important factor herein is that a large part of these false discoveries are based on series of human serum samples, with the accompanying issues of human serum complexity, variability and dynamic range, next to biases resulting from poor study design, inappropriate sample selection and statistical issues such as overfitting of data.

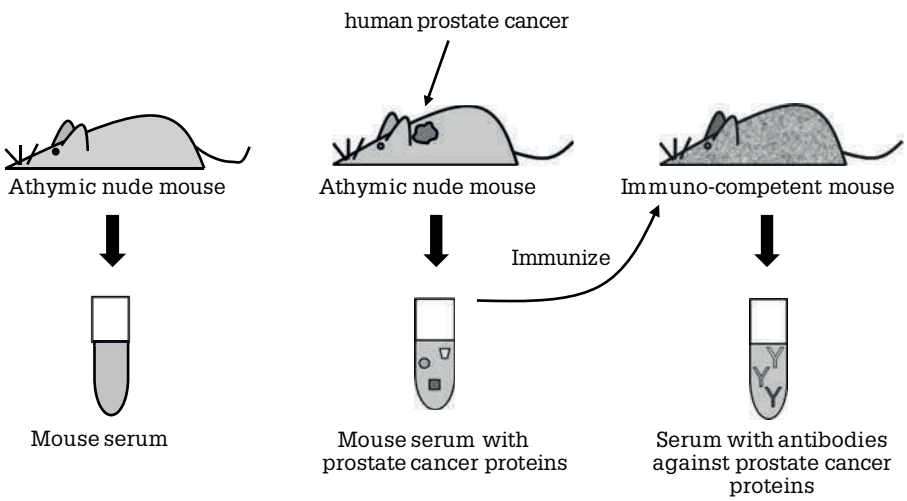

Figure 1. The two xenograft-based biomarker discovery platforms utilized in this thesis. After inoculation with a human prostate cancer graft, PCa proteins are secreted into the mouse bloodstream (middle mouse). By comparing serum of tumour-bearing mice (middle mouse) with serum of non-tumour-bearing mice (left mouse), PCa-derived proteins can be identified (Chapter 2). In addition, an immune-competent congenic mouse (right mouse) can be immunized with serum drawn from a tumour-bearing mouse (middle mouse) to generate an antibody response against human PCa-derived proteins (Chapter 3).

To bypass these issues we have chosen the xenograft model system as a platform for the discovery of new biomarkers for PCa[123]. In this system, human prostate tumour fragments are transplanted onto athymic nude mice. We hypothesized that, once securely grafted, these xenografts will shed tumour proteins into the mouse circulation. Thus, a normal nude mouse has only mouse proteins in its circulation, while the serum of a nude mouse bearing a human xenograft contains also human proteins deriving from the prostate tumour (Figure 1). This means that any protein identified as human specific is directly derived from the xenograft itself.

This approach evades the issues associated with comparing large series of human serum samples as described above. There is no detrimental sample variation: a single recipient mouse carrying a PCa xenograft should in principle suffice for the identification of PCa-derived proteins from mouse serum. Given the subsequent limited number of samples needed, a comprehensive and detailed scan of all high and low abundant PCa-derived proteins from a single serum sample can be performed by employing the latest MS technologies. The challenge with respect to the enormous dynamic range of serum protein expression can thus partially be overcome. As has already been reported, this model system is indeed capable of identifying human proteins that are shed into the circulation by human PCa cells[124]. In Chapter 2, 44 tumour-derived proteins were identified utilizing this xenograft model. Amongst these were almost all subunits of the proteasome, a proteolytic protein complex. We hypothesize that their presence reflects the dysregulation of protein synthesis and degradation in cancer cells in contrast to normal cells in which the proteasome complex plays a crucial role in controlling essential cellular functions such as transcription, stress response, cell cycle regulation, cellular differentiation, and DNA repair[143]. Several subunits of the proteasome have indeed been linked to PCa, as well as to other malignancies[241-245]. However, one should keep in mind that the transplanted xenograft is relatively large in size compared to the total blood volume of the nude mouse. Translated to the human situation, this could mean that the number of circulating proteasomes deriving from PCa cells is undetectable against the high background levels of naturally circulating proteasomes. This is illustrated in Chapter 3, which showed no significant differences in concentrations of the PSMA1 proteasome subunit in men with or without PCa. In contrast, elevated serum levels of the 20S proteasome have been shown in patients with other solid tumours, such as breast cancer and renal cell carcinoma[241,242]. Nevertheless, the possibility still exists that specific posttranslational processes such as glycosylation created a more PCa specific proteasome isoform. Recently, the presence of a proteasome β subunit exclusively present in thymic cortical epithelial cells was discovered and shown to be useful in differentiating thymic carcinomas[245].

Another question that has to be elucidated is which of the identified PCa-derived proteins are specifically secreted by cancer cells and not by normal prostate cells. Most likely, the majority of these proteins are also secreted under normal circumstances, but in cancer cells the proportion of secreted proteins might differ, resulting in higher serum levels of specific proteins. However, it might well be that indeed some of the identified proteins are specifically secreted by PCa cells, and are not secreted by normal prostate tissue. Also, the disruption of the normal tissue architecture in PCa may play an important role, such as is the case for the elevated serum levels of PSA in PCa patients. A first start to elucidate some of these questions is a control experiment, in which human proteins are identified in the serum of mice inoculated with normal prostate tissue.

Prostate cancer-derived proteins in the xenograft model systems are part of exosomes

Another observation is that about half of the discovered tumour-derived proteins in Chapter 2 are not formally known as secreted proteins but are annotated as being cytoplasmatic. This remarkable observation put us on the path of exosomes. Exosomes are small membrane vesicles, secreted by virtually every cell type, including tumour cells[126]. They are formed in multivesicular bodies by inward budding, thereby encapsulating cytoplasmic components (Figure 2)[126,127]. The exact function of exosomes in tumour cells has still to be elucidated, but is expected to relate to roles in cell-to-cell crosstalk, tumour-stromal interaction, protein degradation and antigen presentation[126,127].

A recently published study reported that exosomes which are secreted by tumour cells can inhibit the binding of tumour-reactive antibodies to tumour cells. In this way exosomes might help tumour cells to escape from immune surveillance[246].

An in-depth analysis of the PCa-secreted exosomes was performed in Chapter 2. After isolation of exosomes derived from PCa cells, these were analysed for their protein content by MS. This showed that indeed a large portion of the proteins inside exosomes could be annotated as cytoplasmic, next to the presence of cell membrane-associated proteins. This finding was confirmed in a recent review by György *et al.* describing the subcellular localisation of proteins identified in exosomes from six different studies[247]. It has been hypothesized that inside exosomes these cytoplasmic proteins are protected from degradation in human plasma, hereby enabling these cytoplasmic proteins to act distant from the site of their production. Another fascinating finding was the presence of almost all proteasome subunits amongst the identified exosomal proteins. This observation was recently confirmed

in recent report from Lai *et al.* investigating the protein content in exosomes derived from mesenchymal stem cells, showing the presence of all seven α and β subunits of the 20S proteasome inside exosomes[248]. The presence of proteasomes inside exosomes might point towards the occurrence of specific proteolytic processes inside exosomes, which is suggested by the finding of a distinct 30-kDa ENO1 band specifically present in the exosome fraction as described in Chapter 2. Unfortunately, our attempts to specifically identify this 30-kDa ENO1 band by MS/MS were not successful.

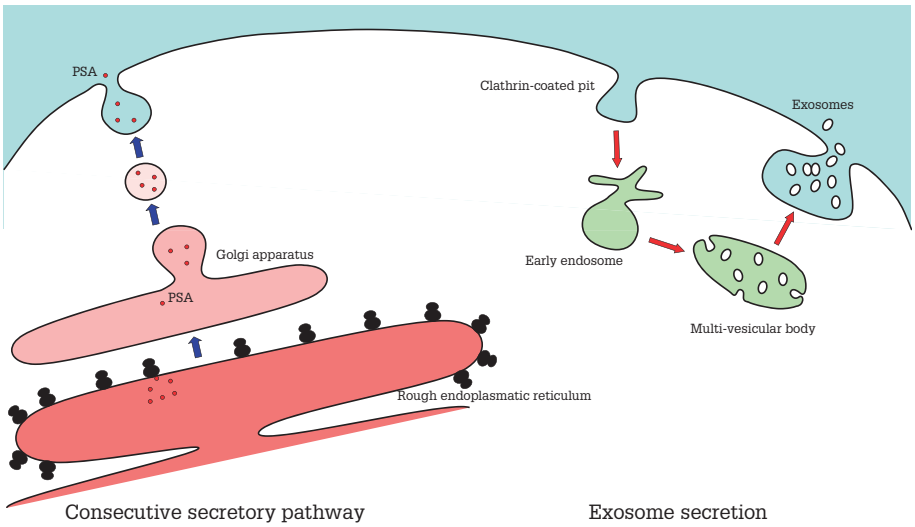

Figure 2. In the consecutive secretory pathway proteins such as PSA (prostate-specific antigen) are formed in the rough endoplasmatic reticulum, a membrane-bound compartment in the cell. The proteins are then transferred to the many compartments of the Golgi apparatus and finally end up in a vesicle that fuses with the cell plasma membrane, releasing the proteins outside of the cell. In contrast to the consecutive secretory pathway, exosomes are formed in multivesicular bodies by inward budding, thereby encapsulating cytoplasmic proteins, such as cytoskeletal proteins, kinases, metabolic enzymes, and also RNA.

In addition to analysing the exosomal protein content, we specifically identified the TMPRSS2:ERG gene fusion transcript inside exosomes from PCa cell cultures. Surprisingly, this is also the case in the human situation. In a small pilot study, Nilsson *et al.* were able to detect the TMPRSS2:ERG fusion mRNA in exosomes isolated in urine from men with PCa[249]. Although the exact function of mRNA inside exosomes remains speculative, it has been shown that the exosomal RNA is not a

random sample from the cellular RNA pool, as certain mRNA transcripts are enriched up to several 100-fold in exosomes, suggesting a specific sorting mechanism[250,251]. Additionally, it has been shown that the number of exosomes is elevated in the circulation of cancer patients compared to normal controls[252,253].

In conclusion, exosomes represent a fingerprint of the cancer cell, and as exosome concentrations are elevated in the circulation of cancer patients, exosomes present biomarker treasure chests. By specific enrichment for PCa-derived exosomes, utilizing tissue-specific membrane proteins present on exosomes, also the challenge of the dynamic range problem associated with the search for novel biomarkers might be overcome. As it has been proven that exosomes are relatively stable under multiple cycles of freezing and thawing, large scale purification of PCa-derived exosomes from biobank samples would be feasible[254]. Up to date, several studies have already been performed investigating the clinical usefulness of exosomes in PCa detection and prognosis. However preliminary, these studies have shown that it is indeed possible to identify, stage and monitor men with PCa based on their exosome-associated biosignature[255-258].

Regarding future exosome research, a stable high-throughput assay should be developed to analyse exosomal protein content, allowing for a first large scale validation of possible exosomal protein markers. Then, a robust and clinical applicable assay should be developed, to isolate and analyse PCa-derived exosomes from men without and men with different stages of PCa, in order to facilitate the entry of a novel biomarker into routine clinical practice.

In addition, investigations regarding the possible functional role of exosomes might elucidate important mechanisms, such as cancer metastasis. Recently, it was shown that melanoma-derived exosomes harbour specific messenger proteins, enhancing metastatic invasion by directing bone marrow-derived cells toward a prometastatic phenotype, initiating a metastatic niche[259]. In turn, this might also have important consequences from a therapeutical point of view, regarding the prevention and prediction of metastases.

A unique xenograft-based immunization method detects novel prostate cancer biomarkers.

The approach taken in Chapter 2 is far more specific and has less variability than analysing hundreds of human serum samples. However, its results are still limited by the protein complexity of mouse serum itself and the resolution and mass accuracy of current mass spectrometers. In Chapter 3, we utilized the highly sensitive and specific mouse immune system to discover novel biomarkers for PCa. We developed

a xenograft-based biomarker discovery platform, in which immuno-competent mice are immunized with serum from PCa xenograft-bearing nude mice (Figure 1). In this system, the donor and recipient mice are congenic, so only the human PCa-derived proteins will specifically generate immunoglobulin biomarkers. Subsequently, a protein microarray-based method was utilized to profile the antibody production and identify PCa xenograft-derived proteins.

This approach resulted in the identification of 99 proteins to which an antibody response was observed. Amongst these were proteasome subunit PSMA1, ACY1, and two members of the TAM receptor family, TYRO3 and MERTK. After validation of these proteins by ELISA in serum samples from xenografted mice, a prevalidation was performed on series of human serum samples. Significantly different concentrations of several of the identified proteins were shown, in men with or without PCa and in men with PCa metastasis or PCa recurrence, proving that this unique xenograft-based immunization method is indeed capable of detecting possible novel PCa biomarkers.

However, as so many possible biomarkers are identified in the discovery phase by utilizing the approach in Chapters 2 and 3, it is virtually impossible to assess the value of all discovered proteins in a validation phase. To solve this dilemma, a selection was made based on imprecise criteria, such as the number of identified peptides of a specific protein, ProtoArray signal intensities, the presence of a protein in a specific cellular compartment, or results of previously published data. In this way, valuable candidates may be overlooked. Thus, high-throughput assays are needed to validate all identified candidates in a limited amount of time and effort.

Despite these dilemmas and challenges, both xenograft-based biomarker discovery platforms provide an important addition to existing means of biomarker discovery. In the future, a high-throughput assay should be developed to analyse the presence of all possible markers in a first validation series, so the most promising candidates can be selected. Then, further large scale identification in multi-centre series is needed to assess if any of the discovered proteins holds its promise as a novel biomarker. In addition, by investigating the role in PCa tumorigenesis and metastasis of the proteins identified by the xenograft-based biomarker discovery platforms, biologically related proteins may be identified, which in turn could represent novel biomarkers. Last of all, this might also have implications for PCa therapy. For example, it has been shown that inhibition of MERTK and AXL promotes apoptosis and inhibits tumour growth[260-262].

Validation of isoforms of prostate-specific antigen as diagnostic markers

As mentioned previously, a disappointingly low number of novel biomarkers have reached the clinic during the last decades. Often, a novel candidate is presented with great promise, but later on subsequent validation studies show inconsistent or even contradictionary results. There are multiple possible explanations which can clarify the discrepancies encountered in validation studies, varying from methodological differences, poor study design, the use of inappropriate statistical tests, insufficient sample sizes and the use of unstandardised assays. On the other hand, if subsequent validation studies do show a consistent but limited improvement in diagnostic or prognostic value, chances of clinical incorporation are small, in sharp contrast with small improvements in the therapeutic field, such as an increased survival of several weeks to months[240]. A possible explanation for this phenomenon is that clinicians tend to over-treat patients, rather than incorporating biomarkers with less than perfect prediction into their clinical decision making.

During the last decades, several initiatives have been launched to improve the quality of reporting on validation of novel biomarkers. Examples are the STARD initiative (Standards for Reporting of Diagnostic Accuracy), presenting a checklist of 25 items to ensure reporting of all relevant information, and the REMARK recommendation (Reporting recommendations for tumour Marker prognostic studies), a guideline to encourage transparent and complete reporting[263,264].

In Chapter 4, a retrospective multi-centre validation study is described. In this study, the PSA isoforms p2PSA and BPHA were analysed for their capacity in PCa detection as well as prognostic power. It was shown that p2PSA and, moreover, %p2PSA and phi could have additional value with respect to tPSA and %fPSA in PCa detection within the tPSA range of 2– 10 ng/ml, by significantly increasing the predictive value and specificity for PCa. In contrast, %p2PSA and phi seemed to have limited additional value in identifying men with aggressive PCa. Several very recent reports have confirmed that %p2PSA, and especially phi, are superior over tPSA and fPSA regarding predictive value in PCa detection. Studies by Stephan, Lazzeri, Catalona, Guazzoni and Le *et al.* showed a more than doubled specificity and significantly greater predictive value of %p2PSA and moreover phi in initial PCa screening, compared to tPSA and fPSA[265-269]. Amongst these were several prospective and multi-centre studies. In addition, the majority of these reports showed a correlation between higher levels of phi and the presence of PCa with a GS ≥7. A systematic review and meta-analysis by Filella *et al.*, analysing twelve studies, confirmed these results on the diagnostic performance as well as on the relationship of phi and %p2PSA with PCa aggressiveness[270].

Interestingly, reports investigating the performance of phi compared to PCA3 and TMPRSS2:ERG in initial PCa screening showed similar or superior results for phi compared to the latter two[271-273].

So, as randomized controlled trials are awaited, preferably performed according to a biomarker reporting checklist or guideline, there are very strong indications that %p2PSA and especially phi are significantly better predictors for the presence of PCa than tPSA alone within a tPSA range of 2-10 ng/ml. As a result, the implementation of phi in routine clinical practice might result in fewer unnecessary prostate biopsies, reducing healthcare costs, as has been calculated in a recent report[274]. Recently, phi was granted premarket approval by the FDA for men 50 years and older with a tPSA value between 4 and 10 ng/ml and a digital rectal exam with no suspicion of cancer (http://www.accessdata.fda.gov/cdrh_docs/pdf9/p090026a.pdf). This is a first step into the implementation of phi in current clinical practice, however the most appropriate cut-off levels for phi and %p2PSA should still be established.

Total prostate-specific antigen standardisation issues

As mentioned previously, also assay standardisation issues are related to discrepancies resulting from biomarker validation studies. Ideally, there should be only one assay platform from one manufacturer which is used worldwide to measure a certain biomarker. However, as there are huge financial interests in screening for specific diseases, there are often multiple manufacturers developing their own commercial assays. The resulting interassay variability can at least partly be overcome by using a specific calibration standard, and ideally this standard should be agreed upon before widespread clinical use of a specific assay, however this is rarely implemented. For example, it has taken more than a decade after its introduction before a calibration standard was adopted by the WHO for the tPSA assay, indeed decreasing interassay variabilities. However, by introducing a novel calibration standard long after widespread clinical use of a specific test, confusion is created as reported values will shift for certain assay platforms.

In Chapter 5, it is shown that for the Beckman Coulter tPSA assay, the introduction of the WHO reference standard resulted in a 20% decrease in tPSA concentrations with a subsequent decrease in the number of prostate biopsies and detected cancers. However, in the light of PCa overdetection and overtreatment it may be discussed if this is actually a negative or positive consequence of standardisation, as the great majority of the missed cancers were low-grade cancers. A more recent study from Fileé *et al*. produced nearly identical results[275]. To conclude, as long as significant

differences in tPSA values are reported by different assay platforms, physicians should be aware which assay platform is applied in their local hospital as well as which calibration method is used, before making possible far-reaching decisions at a patient level.

EPILOGUE

Although results from prostate cancer screening studies are still under debate, screening for prostate cancer will always be carried out, either in an opportunistic or more organised way. This results from the reality that a substantial number of men bear in mind the possibility of being the one patient with an aggressive form of prostate cancer, which could be cured, but only if discovered in an early phase. Instead, it is more likely that they belong to the larger group of men with insignificant disease that will suffer from the consequences of overtreatment of indolent prostate cancer. The longer it takes before novel markers are introduced into clinical practice, the more men will suffer from the confusion that has resulted from the present studies evaluating screening for prostate cancer. A first step in improving the detection of prostate cancer could be the implementation of phi into routine clinical practice, integrating phi with existing markers and prostate cancer risk calculators. With relatively little effort and costs, this would lead to a significantly better performance in prostate cancer detection. Furthermore, it is clear that the biggest challenges lie now in the field of biomarker validation, as this thesis shows that the presented xenograft-based biomarker platforms are able to circumvent some of the major issues associated with contemporary biomarker discovery. The first challenge now is to develop novel high throughput methods to assess and validate the huge number of possible biomarkers in a large population of patients and controls.

SUMMARY

In the Netherlands, 10,391 new cases of prostate cancer (PCa) were diagnosed in 2010. This makes PCa the most common malignancy in men (source: Dutch Cancer Registry). To detect PCa in an early stage, prostate-specific antigen (PSA), which can be detected in blood, is frequently utilized. However, as PSA is not PCa specific, elevated levels can also be detected in men with benign prostate hyperplasia (BPH). In addition, a low PSA value does not rule out the presence of PCa. Thus, PSA should rather be regarded as a risk spectrum of PCa. A definitive diagnosis can only be made after taking prostate biopsies, an invasive procedure with associated morbidity. Also, the prognostic value of PSA is limited, as it is not unambiguously correlated to PCa aggressiveness. Thus, as an indirect consequence of utilizing PSA to identify men at risk of being diagnosed with PCa, there is a substantial overdiagnosis and overtreatment of PCa. A novel biomarker for the detection and prognosis of PCa could limit these significantly. Ideally, this biomarker should be able to differentiate between men with or without PCa, differentiate between indolent and aggressive disease, and be suitable for follow-up after treatment. Most likely, not one marker will possess all these characteristics, but a panel of carefully selected markers probably will be able to fulfil these criteria. Without doubt, PSA will be one of these, as physicians are so familiar to making PSA-based clinical decisions.

There are several challenges that should be met and numerous strategies that can be followed discovering novel markers. A frequently utilized approach is based upon comparing serum samples of large groups of men with PCa to the normal population. However, this approach is seriously hampered by the enormous variability of protein content among men, the often limited sample availability, and the time-consuming and laborious analyses that would arise from such an approach. In addition, due to the vast dynamic range in protein concentrations in human serum and the dominance of a small number of high abundant proteins, the presence of low abundant proteins, such as possible novel biomarkers, might be masked.

To bypass these issues we based our approach on a xenograft mouse model for PCa. In this system, human prostate tumour fragments are transplanted onto athymic nude mice. We hypothesized that once securely grafted, these xenografts will shed tumour proteins into the mouse circulation. Thus, a normal nude mouse has only mouse proteins in its circulation, while the serum of a nude mouse bearing a human xenograft contains also human proteins. Subsequently, these tumour-derived and human specific proteins can be identified utilizing mass spectrometry (MS). As

there is no detrimental sample variation, a single recipient mouse carrying a PCa xenograft should in principle suffice for the identification of PCa-derived proteins from mouse serum. In addition, by mimicking the human situation, the xenograft model is able to specifically expose tumour-secreted proteins, as is the case for most tumour markers applied in the clinic today.

In Chapter 2, the utilization of this xenograft mouse model led to the identification of 44 PCa-derived proteins. As expected, part of these proteins have been annotated as being actively secreted by cells. However, a large portion of the identified proteins has not been described as being secreted by cells, but has been annotated as being cytoplasmic. We hypothesised that the presence of these proteins in the mouse circulation results from the secretion of exosomes. These are small membrane vesicles, secreted by virtually every cell type, containing content from the cytoplasmic compartment. A subsequent in-depth analysis of the proteins present in exosomes secreted from PCa cell lines resulted in the identification of almost exclusively cytoplasmic proteins, amongst which were several of the proteins that had earlier been identified in the circulation of xenografted mice. In addition, analyses of exosomal RNA resulted in the identification of the PCa specific TMPRSS2:ERG gene fusion. So, we conclude that PCa-derived exosomes are ideal candidates for the discovery of novel biomarkers for PCa.

In Chapter 3, this xenograft model was further exploited for the discovery of novel biomarkers. By injecting immune-competent mice with serum drawn from PCa xenograft-bearing nude mice, we hypothesized that an immune-response would be generated against human PCa-derived proteins. A protein microarray based approach was subsequently utilized to profile the antibody production and thus indirectly identify PCa xenograft-derived antigens. Amongst 99 other proteins, this resulted in the identification of TYRO3, AXL, MERTK, ACY1 and PSMA1, of which the first three were pre-validated in series of human serum samples. This pre-validation showed significantly different concentrations of the identified proteins in men with or without PCa, and in men with PCa metastasis or PCa recurrence, hereby proving that this unique xenograft-based immunization model is indeed capable of detecting novel PCa biomarkers.

In Chapter 4, we evaluated the additional value of the PSA-isoforms p2PSA and BPHA, both part of the free PSA (fPSA) fraction, with respect to PCa detection. In addition, the value of phi (prostate health index, calculated as (p2PSA/fPSA) x $\sqrt{}$(tPSA)) was investigated. This multicenter study included 756 serum samples of men with or without PCa and a total PSA (tPSA) concentration between 2 and 10 ng/ml, deriving from the European Randomised Study of Screening for Prostate Cancer (ERSPC) and the Urology Department of Innsbruck Medical University. This study showed a significant increase in PCa predictive value and specificity of phi (31 versus

10%) compared to tPSA. Also, %p2PSA (p2PSA/fPSA) performed significantly better than tPSA. A multivariate model predicting the presence of PCa was significantly improved after the addition of p2PSA. Thus, we conclude that the introduction of %p2PSA and moreover phi into routine clinical practice will significantly improve PCa detection, thereby limiting the number of negative prostate biopsies.

Currently, several dozens of different assays are available to measure tPSA concentrations in human serum. As this has resulted in significant inter-assay differences, the World Health Organization (WHO) introduced the WHO 96/670 calibration standard. However, it is anticipated that replacement of the original calibration standard with the WHO 96/670 calibration might lead to significant changes in tPSA results, with serious consequences for cut-off points for taking prostate biopsies, and subsequent changes in PCa detection rates.

In Chapter 5, the consequences of the introduction of the WHO 96/670 calibration for the Beckman Coulter tPSA assay were evaluated. It is shown that as a result of introducing the WHO 96/670 calibration standard, reported tPSA concentrations decreased by approximately 20% for the Beckman Coulter tPSA assay. Without correction of the applied tPSA thresholds for prostate biopsies, this results in a significant decrease in PCa detection rates. Hence, physicians should be aware which calibration method is utilized in their local hospital, and carefully assess which thresholds are used for taking prostate biopsies.

SAMENVATTING

In 2010 werden in Nederland 10.391 nieuwe gevallen van prostaatkanker vastgesteld. Hiermee is prostaatkanker de meest voorkomende vorm van kanker bij mannen (bron: Nederlandse Kankerregistratie). Om prostaatkanker in een vroeg stadium op te sporen wordt veelvuldig gebruik gemaakt van het prostaat specifiek antigeen (PSA), dat in het bloed te bepalen is. Nadeel van de PSA test is echter dat deze niet specifiek is voor prostaatkanker en ook een verhoogde uitslag kan geven indien er alleen sprake is van een goedaardige prostaatvergroting. Tevens kan bij een lage PSA waarde de aanwezigheid van prostaatkanker niet worden uitgesloten. De PSA waarde geeft dus alleen een risico inschatting van de kans op de aanwezigheid van prostaatkanker weer. Daarnaast is de prognostische waarde van PSA beperkt, aangezien de hoogte hiervan niet eenduidig is gerelateerd aan de agressiviteit en bijbehorende prognose van de gevonden prostaattumor. De definitieve aan- of afwezigheid van prostaatkanker valt alleen vast te stellen middels het nemen van prostaatbiopten, een invasieve procedure met de bijbehorende morbiditeit. Als gevolg hiervan is er sprake van een enorme overdiagnose en overbehandeling van prostaatkanker. Een nieuwe biomerker voor de detectie en prognose van prostaatkanker zou dit probleem significant kunnen beperken. De ideale merker zou in een vroeg stadium in staat moeten zijn te differentiëren tussen de aan- of afwezigheid van prostaatkanker, zou wat over de agressiviteit van de tumor moeten zeggen en geschikt moeten zijn voor regelmatige follow-up. Waarschijnlijk is er niet één merker die aan al deze criteria voldoet, maar met een panel van verschillende merkers is dit mogelijk wel te bereiken. Tevens valt het niet te verwachten dat door een nieuwe merker het PSA uit de kliniek verdwijnt, gezien de vertrouwdheid en ervaring van clinici met het nemen van medische beslissingen op basis van PSA uitslagen.

Bij het zoeken naar nieuwe merkers zijn er een aantal uitdagingen en strategieën. Een mogelijke aanpak is het vergelijken van grote groepen patiënten met prostaatkanker met de normale populatie. Lastig hierbij is de enorme variabiliteit binnen patiënten en controles, de vaak beperkte aantallen samples die beschikbaar zijn en de langdurige analyses die nodig zijn om nieuwe merkers te detecteren. Verder zijn er zoveel verschillende eiwitten in serum aanwezig dat die de aanwezigheid van eiwitten die in zeer lage concentraties voorkomen, zoals waarschijnlijk nieuwe merkers, verhullen. Om deze problemen te omzeilen kan men ook gebruik maken van een muismodel. Hierbij wordt gebruik gemaakt van muizen met een niet-

werkend immuunsysteem waarop humane prostaattumoren kunnen groeien. Met behulp van massa spectrometrie kunnen vervolgens de eiwitten die circuleren in het bloed van deze muizen worden geanalyseerd. Het bloed van een muis zonder tumor zal alleen 'muizen-eiwitten' bevatten, het bloed van de muis met tumor zal daarnaast mogelijk ook eiwitten bevatten afkomstig van de prostaattumor. Deze eiwitten onderscheiden zich van de 'muizen-eiwitten' doordat ze als humaan specifiek kunnen worden herkend. Voordeel van dit muismodel is dat er hierbinnen nauwelijks variatie is, aangezien alle muizen genetisch identiek zijn en er steeds gebruikt gemaakt kan worden van dezelfde tumor, waarvan de karakteristieken bekend zijn. Een ander voordeel van deze aanpak is dat de meeste merkers die in de kliniek gebruikt worden, direct worden uitgescheiden door de tumor, een situatie die met het muismodel wordt nagebootst.

In hoofdstuk 2 werden met behulp van dit muismodel 44 eiwitten gevonden die afkomstig waren van de verschillende prostaattumoren. Deze eiwitten bestaan, zoals verwacht, voor een deel uit eiwitten waarvan het reeds bekend is dat ze actief door cellen worden uitgescheiden. Een opmerkelijke bevinding is echter dat er daarnaast ook een groot aantal eiwitten werd geïdentificeerd die normaal niet door cellen worden uitgescheiden, zogenaamde cytoplasmatische eiwitten. Mogelijk zijn deze in het bloed terechtgekomen als gevolg van uitscheiding in exosomen, zeer kleine blaasjes die door cellen worden uitgescheiden vanuit het cytoplasma. Nadere analyse van exosomen afkomstig van prostaatkankercellen toonde aan dat vrijwel alle eiwitten die hierin gevonden werden normaal gesproken alleen in het cytoplasma aanwezig zijn en niet actief worden uitgescheiden. Verschillende van deze eiwitten werden eerder teruggevonden in het bloed van muizen met prostaatkanker. Tevens werd in deze exosomen op RNA niveau het prostaatkanker specifieke fusiegen TMPRSS2:ERG aangetoond. Al met al lijken exosomen dus ideale kandidaten voor de ontdekking van nieuwe merkers voor prostaatkanker.

In hoofdstuk 3 wordt op een andere manier gebruik gemaakt van het muismodel. Door muizen met een intact afweersysteem te immuniseren met bloed afkomstig van muizen met een humane prostaattumor werd een antilichaam respons opgewekt tegen eiwitten afkomstig van deze prostaattumor. Deze eiwitten konden vervolgens worden geïdentificeerd door eiwit microarrays te incuberen met bloed van geïmmuniseerde muizen. Dit leverde onder andere de identificatie op van de eiwitten TYRO3, AXL, MERTK, ACY1 en PSMA1, waarvan de eerste drie werden gevalideerd in humaan serum. Hierbij werden inderdaad significante verschillen gevonden tussen verschillende groepen van mannen met en zonder prostaatkanker. Ook deze methode lijkt dus in staat tot het vinden van nieuwe merkers voor prostaatkanker, hoewel grootschalige validatie van mogelijke merkers nog moet plaatsvinden.

In hoofdstuk 4 werd de additionele waarde van de PSA isovormen p2PSA en BPHA naast tPSA (totaal PSA) en fPSA met betrekking tot de detectie van prostaatkanker onderzocht. Deze PSA isovormen maken onderdeel uit van de vrije PSA fractie (fPSA). Tevens werd de bruikbaarheid van phi (prostate health index, (p2PSA/fPSA) x √(tPSA)) onderzocht. In totaal werden 756 serum samples geanalyseerd van mannen met en zonder prostaatkanker en een tPSA waarde tussen de 2 en 10 ng/ml. Het betrof hier een multicenter studie met samples afkomstig van de European Randomised Study of Screening for Prostate Cancer en de Medische Universiteit van Innsbruck. Uit de resultaten blijkt dat phi de beste voorspeller is voor de aanwezigheid van prostaatkanker, significant beter dan tPSA. Ook de specificiteit van phi is significant beter dan die van tPSA (31 versus 10%). Ook %p2PSA (p2PSA/fPSA) presteerde significant beter dan tPSA. Bij multivariaat analyse zorgde de toevoeging van p2PSA voor een significante verbetering van het voorspellende model. De invoering van phi in de dagelijkse klinische praktijk zou dus de detectie van prostaatkanker kunnen verbeteren en hierdoor tevens het aantal negatieve prostaatbiopten kunnen beperken.

Wereldwijd worden er tientallen platformen van verschillende fabrikanten gebruikt om de waarde van tPSA in serum te kunnen bepalen. Aangezien deze elk hun eigen kalibratie methode gebruiken leidt dit tot substantiële verschillen in tPSA uitslagen. Om deze verschillen te beperken werd door de World Health Organization (WHO) een kalibratie standaard in het leven geroepen, de WHO 96/670. Vervanging van de originele kalibratie door de WHO 96/670 kalibratie standaard zou echter kunnen leiden tot een verschuiving in tPSA waarden, met gevolgen voor afkappunten voor het nemen van prostaatbiopten, weer resulterend in mogelijke veranderingen in prostaatkankerdetectie. In hoofdstuk 5 werden de gevolgen van de introductie van de WHO 96/670 kalibratie standaard voor de tPSA bepaling van Beckman Coulter onderzocht. Hieruit blijkt dat bij toepassing van de WHO 96/670 kalibratie de tPSA waarden 20% lager uitvallen. Wanneer de afkappunten voor het nemen van prostaatbiopten vervolgens niet adequaat worden aangepast resulteert dit ook in een vermindering van het aantal gedetecteerde prostaattumoren. Hoewel het overgrote deel hiervan relatief 'gunstige' prostaattumoren betreft is het van groot belang dat clinici zich ervan bewust zijn welke tPSA bepaling er in hun ziekenhuis wordt gebruikt en welke kalibratie standaard er wordt toegepast.

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Work

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Awards

- Scholar-in-Training Award, American Association for Cancer Research, Innovations in Prostate Cancer Research, San Francisco, 2006
- Best poster on 'New Biomarkers for Prostate Cancer', 22nd Annual Congress of the European Association of Urology, Berlin, 2007
- Best poster on 'Improvements on the diagnosis and prognosis of prostate cancer', 23nd Annual Congress of the European Association of Urology, Milan, 2008

Publications

Elevated levels of d-dimer and fragment 1+2 upon central venous catheter insertion and factor V Leiden predict subclavian vein thrombosis
F.H. Jansen, H.M. van der Straaten, M. Roest, F. Haas, Ph.G. de Groot, R. Fijnheer
Haematologia; 2005; 90: 499-504

Risicofactoren bij recidiverende urineweginfecties
F.H. Jansen, E.R. Boevé
Nederlands Tijdschrift voor Urologie; 2006; 7: 194-99

PSA isovormen verbeteren de prognostische waarde van prostaatkanker biopten, maar kunnen deze niet vervangen
F.H. Jansen, R.H.N. van Schaik, J. Kurstjens, M.F. Wildhagen, M. Roobol, C.H. Bangma
Nederlands Tijdschrift voor Klinische Chemie en Laboratoriumgeneeskunde 2007; 32: 259-261

Recidiverende urineweginfecties bij vrouwen: optimaliseren van het beleid.
E.P. van Haarst, F.H. Jansen, T. Wiersma
Nederlands Tijdschrift voor Urologie; 2008;2:71-76

The clinical impact of the new prostate-specific antigen WHO standardization on prostate biopsy rates and prostate cancer detection
F.H. Jansen, M. Roobol, C.H. Bangma, R.H.N. van Schaik
Clinical Chemistry; 2008;54: 1999-2006

Prostate specific antigen: an update
C.H. Bangma, F.H.Jansen, M. Roobol
International Handbook of Prostate Cancer, 2008, 3d edition; Chapter 3b: 73-81

Exosomal secretion of prostate cancer derived proteins: exosomes as biomarker treasure chests
F.H. Jansen, J. Krijgsveld, A. van Rijswijk, R. Willemsen, T.M. Luider, G. Jenster
Molecular and Cellular Proteomics; 2009; 8: 1192-205

Screening for prostate cancer in 2008 II: the importance of molecular subforms of PSA and tissue kallikreins
Flip.H. Jansen, Monique Roobol, Guido Jenster, Fritz H. Schröder, Chris H. Bangma
European Urology; 2009; 55: 563-74

Niet-antibiotische profylaxe bij recidiverende urineweginfecties
F.H. Jansen, E.P. van Haarst
Nederlands Tijdschrift voor Urologie; 2009; 5: 125-129

p2PSA in combination with total PSA and free PSA improves diagnostic accuracy in prostate cancer detection
Flip H. Jansen, Ron H.N. van Schaik, Joep Kurstjens, Wolfgang Horninger, Helmut Klocker, Jasmin Bektic, Mark F. Wildhagen, Monique Roobol, Chris H. Bangma, Georg Bartsch
European Urology; 2010; 57: 921-7

Profiling of antibody production against xenograft-released proteins by protein microarrays discovers prostate cancer markers
F.H. Jansen, A. van Rijswijk, W. Teubel, W.M. van Weerden, S. Reneman, G.J. van den Bemd, M. Roobol, C.H. Bangma, F.J.T. Staal, G. Jenster
Journal of Proteome Research; 2012 Feb 3;11(2):728-35

Presentations

Elevated levels of d-dimer and fragment 1+2 upon central venous catheter insertion and factor V Leiden predict subclavian vein thrombosis; F.H. Jansen, H.M. van der Straaten, M. Roest, F. Haas, Ph.G. de Groot, R. Fijnheer
- Nederlandse vereniging voor Haematologie, Lunteren, 2002
- International Society on Thrombosis and Haemostasis, Birmingham, 2003

Oncological outcome and treatment related toxicity in patients with localised prostate cancer following HDR-^{192}IR brachytherapy combined with external beam radiotherapy. F.H. Jansen, M.F. Wildhagen, W.J. Kirkels, P.P. Jansen, C.H. Bangma, B.J. Chaplin
- Symposium 'Behandeling lokaal prostaatcarcinoom', Rotterdam, 2005

DR -^{192}IR brachytherapie in combinatie met uitwendige radiotherapie bij het lokaal beperkt prostaatcarcinoom: toxiciteit en PSA verloop; F.H. Jansen, M.F. Wildhagen, W.J. Kirkels, P.P. Jansen, C.H. Bangma, B.J. Chaplin
- Najaarscongres Nederlandse vereniging voor Urologie, Utrecht, 2005

Xenograft-based discovery of biomarkers for prostate cancer; F.H. Jansen, J. Krijgsveld, G.J. van den Bemd, A. van Rijswijk, J. Demmers, G. Jenster
- American Association for Cancer Research, Innovations in Prostate Cancer Research, San Francisco, 2006

Evaluation of the Beckman Coulter Access Immunoassay for prostate-specific antigen isoforms in the prediction of cancer recurrence and aggressiveness; F.H. Jansen, R.H.N. van Schaik, J. Kurstjens, M.F. Wildhagen, M. Roobol, C.H. Bangma
- 17th IFCC - FESCC European Congress of Clinical Chemistry and Laboratory Medicine, Amsterdam, 2007

PCa detection by PSA isoform markers: a cross-validation study; Flip H. Jansen, Ron H.N. van Schaik, Joep Kurstjens, Wolfgang Horninger, Helmut Klocker, Jasmin Bektic, Mark F. Wildhagen, Monique Roobol, Chris H. Bangma, Georg Bartsch
– P-Mark meeting, Malmö, 2008

The use of -2proPSA and BPSA in prostate cancer detection: external cross-validation of a predictive model; Flip H. Jansen, Ron H.N. van Schaik, Joep Kurstjens, Wolfgang Horninger, Helmut Klocker, Jasmin Bektic, Mark F. Wildhagen, Monique Roobol, Chris H. Bangma, Georg Bartsch
– American Urological Association (AUA) Annual Meeting, Orlando, 2008

De ontdekking van nieuwe prostaatkanker markers door antilichaam productie tegen humaan xenograft-geproduceerde eiwitten; F.H. Jansen, A. van Rijswijk, W. Teubel, W.M. van Weerden, S. Reneman, G.J. van den Bemd, M. Roobol, C.H. Bangma, F.J.T. Staal, G. Jenster
– Voorjaarsvergadering Nederlandse vereniging voor Urologie, Nieuwegein, 2009

De prostate health index: van aanvullende waarde in prostaatkankerdiagnostiek?; F.H. Jansen
– Symposium 'Het Toekomstbestendige Laboratorium' Star-MDC, Rotterdam, 2010

Prostaat zorgstraat; de rol van de prostate health index (phi); F.H. Jansen
– Beckman Coulter Symposium, Utrecht, 2011

Poster presentations

Oncological outcome and treatment related toxicity in patients with localised prostate cancer following HDR-^{192}IR brachytherapy combined with external beam radiotherapy; F.H. Jansen, M.F. Wildhagen, W.J. Kirkels, P.P. Jansen, C.H. Bangma, B.J. Chaplin
– 21st Annual Congress of the European Association of Urology, Paris, 2006

Identification of prostate cancer-derived serum proteins in xenograft-bearing mice; F.H. Jansen, G.J. van den Bemd, J. Krijgsveld, A. van Rijswijk, J. Demmers, G. Jenster
– Fall meeting Netherlands Proteomics Platform, Leiden, 2006
– HUPO 5th Annual World Congress, Long Beach, 2006

Xenograft-based discovery of biomarkers for prostate cancer; F.H. Jansen, J. Krijgsveld, G.J. van den Bemd, A. van Rijswijk, J. Demmers, G. Jenster
– American Association for Cancer Research, Innovations in Prostate Cancer Research, San Francisco, 2006

Search for human prostate cancer markers in serum of xenograft-bearing mice; F.H. Jansen, J. Krijgsveld, G.J. van den Bemd, A. van Rijswijk, J. Demmers, G. Jenster
– 22nd Annual Congress of the European Association of Urology, Berlin, 2007
– Najaarscongres Nederlandse vereniging voor Urologie, Utrecht, 2005

Proteins entrapped in PCa-derived exosomes as a source of novel serum markers; F.H. Jansen, J. Krijgsveld, R. Willemsen, A. van Rijswijk, G. Jenster
– 7th World Basic Urological Research Congress, Dublin, 2007
– Najaarscongres Nederlandse vereniging voor Urologie, Nieuwegein, 2008

Prostate cancer-derived exosomes as novel biomarker treasure chests; F.H. Jansen, J. Krijgsveld, R. Willemsen, T.M. Luider, A. van Rijswijk, G. Jenster
– 23thd Annual Congress of the European Association of Urology (EAU), Milaan, 2008

Exosomes as a tool for prostate cancer biomarker discovery; F.H. Jansen, J. Krijgsveld, R. Willemsen, T.M. Luider, A. van Rijswijk, G. Jenster
– 12th Molecular Medicine Day, Rotterdam, 2008

The clinical impact of the PSA assay WHO calibration on prostate biopsy rates and prostate cancer detection; F.H. Jansen, M. Roobol, C.H. Bangma, R.H.N. van Schaik
– American Urological Association (AUA) Annual Meeting, Orlando, 2008

Function, composition and secretion of prostate cancer derived exosomes; F.H. Jansen, J. Krijgsveld, R. Willemsen, T.M. Luider, M.S. van den Berg, A. van Rijswijk, G. Jenster
– 18th Meeting of the European Society for Urological Research, Barcelona, 2008

Percent (-2)proPSA improves diagnostic accuracy in prostate cancer detection; Flip H. Jansen, Ron H.N. van Schaik, Joep Kurstjens, Wolfgang Horninger, Helmut Klocker, Jasmin Bektic, Mark F. Wildhagen, Monique Roobol, Chris H. Bangma, Georg Bartsch
– 23th Congress of the European Association of Urology (EAU), Stockholm, 2009

The (-2)proPSA/fPSA ratio (%(-2)proPSA) improves diagnostic accuracy for prostate cancer detection; R.H.N. van Schaik, F.H. Jansen, J. Kurstjens, W. Horninger, H. Klocker, J. Bektic, M.F. Wildhagen, M. Roobol, C.H. Bangma, G. Bartsch
– 18th IFCC - EFCC European Congress of Clinical Chemistry and Laboratory Medicine, Innsbruck, 2009

p2PSA in combination with total PSA and free PSA improves diagnostic accuracy in prostate cancer detection; Flip H. Jansen, Ron H.N. van Schaik, Joep Kurstjens, Wolfgang Horninger, Helmut Klocker, Jasmin Bektic, Mark F. Wildhagen, Monique Roobol, Chris H. Bangma, Georg Bartsch
– American Urological Association (AUA) Annual Meeting, San Francisco, 2010

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ABBREVIATIONS

ACY1	aminoacylase 1
AUC	area under the curve
AXL	AXL receptor tyrosine kinase
BPH	benign prostatic hyperplasia
BPHA (or BPSA)	benign prostatic hyperplasia–associated PSA (or 'benign'PSA)
CD9	tetraspanin 29
DRE	digital rectal examination
ELISA	enzyme-linked immunosorbent assay
ENO1	alpha enolase
ERSPC	European Randomized Study of Screening for Prostate Cancer
fPSA	free PSA
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GS	Gleason score
hK2	human kallikrein 2
MERTK	c-mer proto-oncogene tyrosine kinase
MS	mass spectroscopy
PCa	prostate cancer
PC339	androgen insensitive PCa cell line
PC346	androgen sensitive PCa cell line
phi	Prostate Health Index (= (p2PSA/fPSA) x √(tPSA))
pPSA	proPSA
PSA	prostate-specific antigen
PSMA1	proteasome subunit, alpha type, 1
p2PSA	(-2)proPSA
tPSA	total PSA
TRUS	transrectal ultrasound
TYRO3	TYRO3 protein tyrosine kinase
WHO	World Health Organization
%fPSA	fPSA/tPSA

REFERENCES

1. Lee, C. H., Akin-Olugbade, O., and Kirschenbaum, A., Overview of prostate anatomy, histology, and pathology. *Endocrinol Metab Clin North Am* 40 (3), 565 (2011).
2. Lilja, H., A kallikrein-like serine protease in prostatic fluid cleaves the predominant seminal vesicle protein. *J Clin Invest* 76 (5), 1899 (1985).
3. Hara, M., Koyanagi, Y., Inoue, T., and Fukuyama, T., [Some physico-chemical characteristics of " -seminoprotein", an antigenic component specific for human seminal plasma. Forensic immunological study of body fluids and secretion. VII]. *Nihon Hoigaku Zasshi* 25 (4), 322 (1971).
4. Armbruster, D. A., Prostate-specific antigen: biochemistry, analytical methods, and clinical application. *Clin Chem* 39 (2), 181 (1993).
5. Rittenhouse, H. G., Finlay, J. A., Mikolajczyk, S. D., and Partin, A. W., Human Kallikrein 2 (hK2) and prostate-specific antigen (PSA): two closely related, but distinct, kallikreins in the prostate. *Crit Rev Clin Lab Sci* 35 (4), 275 (1998).
6. Wang, M. C., Valenzuela, L. A., Murphy, G. P., and Chu, T. M., Purification of a human prostate specific antigen. *Invest Urol* 17 (2), 159 (1979).
7. Kuriyama, M., Wang, M. C., Papsidero, L. D. et al., Quantitation of prostate-specific antigen in serum by a sensitive enzyme immunoassay. *Cancer Res* 40 (12), 4658 (1980).
8. Papsidero, L. D., Wang, M. C., Valenzuela, L. A. et al., A prostate antigen in sera of prostatic cancer patients. *Cancer Res* 40 (7), 2428 (1980).
9. Papsidero, L. D., Wojcieszyn, J. W., Horoszewicz, J. S. et al., Isolation of prostatic acid phosphatase-binding immunoglobulin G from human sera and its potential for use as a tumor-localizing reagent. *Cancer Res* 40 (8 Pt 2), 3032 (1980).
10. Balk, S. P., Ko, Y. J., and Bubley, G. J., Biology of prostate-specific antigen. *J Clin Oncol* 21 (2), 383 (2003).
11. Magklara, A., Scorilas, A., Stephan, C. et al., Decreased concentrations of prostate-specific antigen and human glandular kallikrein 2 in malignant versus nonmalignant prostatic tissue. *Urology* 56 (3), 527 (2000).
12. Andriole, G. L., Levin, D. L., Crawford, E. D. et al., Prostate Cancer Screening in the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial: findings from the initial screening round of a randomized trial. *J Natl Cancer Inst* 97 (6), 433 (2005).
13. Stamey, T. A., Yang, N., Hay, A. R. et al., Prostate-specific antigen as a serum marker for adenocarcinoma of the prostate. *N Engl J Med* 317 (15), 909 (1987).
14. Mikolajczyk, S. D., Marks, L. S., Partin, A. W., and Rittenhouse, H. G., Free prostate-specific antigen in serum is becoming more complex. *Urology* 59 (6), 797 (2002).

15. Society, American Cancer, What are the key statistics of prostate cancer?, Available at http://www.cancer.org/cancer/prostatecancer/detailedguide/prostate-cancer-key-statistics, (2010).
16. Gronberg, H., Prostate cancer epidemiology. *Lancet* 361 (9360), 859 (2003).
17. Sakr, W. A., Haas, G. P., Cassin, B. F. et al., The frequency of carcinoma and intraepithelial neoplasia of the prostate in young male patients. *J Urol* 150 (2 Pt 1), 379 (1993).
18. Mariotto, A. B., Robin Yabroff, K., Shao, Y. et al., Projections of the cost of cancer care in the United States: 2010-2020. *J Natl Cancer Inst* 103 (2), 117 (2011).
19. Slobbe, LCJ, Smit, JM, Groen, J et al., Cost of Illness in the Netherlands 2007. (RIVM) (2011).
20. McDavid, K., Lee, J., Fulton, J. P. et al., Prostate cancer incidence and mortality rates and trends in the United States and Canada. *Public Health Rep* 119 (2), 174 (2004).
21. Farkas, A., Schneider, D., Perrotti, M. et al., National trends in the epidemiology of prostate cancer, 1973 to 1994: evidence for the effectiveness of prostate-specific antigen screening. *Urology* 52 (3), 444 (1998).
22. Jacobsen, S. J., Katusic, S. K., Bergstralh, E. J. et al., Incidence of prostate cancer diagnosis in the eras before and after serum prostate-specific antigen testing. *JAMA* 274 (18), 1445 (1995).
23. Cremers, R. G., Karim-Kos, H. E., Houterman, S. et al., Prostate cancer: trends in incidence, survival and mortality in the Netherlands, 1989-2006. *Eur J Cancer* 46 (11), 2077 (2010).
24. Catalona, W. J., Smith, D. S., Ratliff, T. L., and Basler, J. W., Detection of organ-confined prostate cancer is increased through prostate-specific antigen-based screening. *JAMA* 270 (8), 948 (1993).
25. Smith, D. S. and Catalona, W. J., The nature of prostate cancer detected through prostate specific antigen based screening. *J Urol* 152 (5 Pt 2), 1732 (1994).
26. Thompson, I. M., Pauler, D. K., Goodman, P. J. et al., Prevalence of prostate cancer among men with a prostate-specific antigen level < or =4.0 ng per milliliter. *N Engl J Med* 350 (22), 2239 (2004).
27. Nam, R. K., Toi, A., Klotz, L. H. et al., Assessing individual risk for prostate cancer. *J Clin Oncol* 25 (24), 3582 (2007).
28. van Vugt, H. A., Kranse, R., Steyerberg, E. W. et al., Prospective validation of a risk calculator which calculates the probability of a positive prostate biopsy in a contemporary clinical cohort. *Eur J Cancer* 48 (12), 1809 (2012).
29. Loeb, S. and Catalona, W. J., Prostate-specific antigen in clinical practice. *Cancer Lett* 249 (1), 30 (2007).

30. Djulbegovic, M., Beyth, R. J., Neuberger, M. M. et al., Screening for prostate cancer: systematic review and meta-analysis of randomised controlled trials. *BMJ* 341, c4543 (2010).
31. Scales, C. D., Jr., Curtis, L. H., Norris, R. D. et al., Prostate specific antigen testing in men older than 75 years in the United States. *J Urol* 176 (2), 511 (2006).
32. Andriole, G. L., Crawford, E. D., Grubb, R. L., 3rd et al., Prostate cancer screening in the randomized Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial: mortality results after 13 years of follow-up. *J Natl Cancer Inst* 104 (2), 125 (2012).
33. Heijnsdijk, E. A., der Kinderen, A., Wever, E. M. et al., Overdetection, overtreatment and costs in prostate-specific antigen screening for prostate cancer. *Br J Cancer* 101 (11), 1833 (2009).
34. Schroder, F. H., Hugosson, J., Roobol, M. J. et al., Prostate-cancer mortality at 11 years of follow-up. *N Engl J Med* 366 (11), 981 (2012).
35. Schroder, F. H. and Roobol, M. J., ERSPC and PLCO prostate cancer screening studies: what are the differences? *Eur Urol* 58 (1), 46 (2010).
36. Shteynshlyuger, A. and Andriole, G. L., Cost-effectiveness of prostate specific antigen screening in the United States: extrapolating from the European study of screening for prostate cancer. *J Urol* 185 (3), 828 (2011).
37. Heijnsdijk, EA; Wever, EM; Auvinen, A; Hugosson, J; Ciatto, S; Nelen, V; Kwiatkowski, M;, Villers, A; Páez, A; Moss, SM; Zappa, M; Tammela, TL; Mäkinen, T; Carlsson, S; Korfage,, IJ; Essink-Bot, ML; Otto, SJ; Draisma, G; Bangma, CH; Roobol, MJ; Schröder, FH; de, and Koning, HJ., Quality-of-life effects of prostate-specific antigen screening. *New England Journal of Medicine* 367 (7), 595 (2012).
38. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. *Clin Pharmacol Ther* 69 (3), 89 (2001).
39. Shariat, S. F., Semjonow, A., Lilja, H. et al., Tumor markers in prostate cancer I: blood-based markers. *Acta Oncol* 50 Suppl 1, 61 (2011).
40. Langeberg, W. J., Isaacs, W. B., and Stanford, J. L., Genetic etiology of hereditary prostate cancer. *Front Biosci* 12, 4101 (2007).
41. Martin, D. N., Starks, A. M., and Ambs, S., Biological determinants of health disparities in prostate cancer. *Curr Opin Oncol* 25 (3), 235 (2013).
42. Williams, H. and Powell, I. J., Epidemiology, pathology, and genetics of prostate cancer among African Americans compared with other ethnicities. *Methods Mol Biol* 472, 439 (2009).
43. Hemminki, K. and Czene, K., Age specific and attributable risks of familial prostate carcinoma from the family-cancer database. *Cancer* 95 (6), 1346 (2002).
44. Ahn, J., Moslehi, R., Weinstein, S. J. et al., Family history of prostate cancer and prostate cancer risk in the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) Study. *Int J Cancer* 123 (5), 1154 (2008).

45. Kalish, L. A., McDougal, W. S., and McKinlay, J. B., Family history and the risk of prostate cancer. *Urology* 56 (5), 803 (2000).
46. Cerhan, J. R., Parker, A. S., Putnam, S. D. et al., Family history and prostate cancer risk in a population-based cohort of Iowa men. *Cancer Epidemiol Biomarkers Prev* 8 (1), 53 (1999).
47. Colloca, G. and Venturino, A., The evolving role of familial history for prostate cancer. *Acta Oncol* 50 (1), 14 (2011).
48. Nakagawa, H., Akamatsu, S., Takata, R. et al., Prostate cancer genomics, biology, and risk assessment through genome-wide association studies. *Cancer Sci* 103 (4), 607 (2012).
49. Lundwall, A. and Lilja, H., Molecular cloning of human prostate specific antigen cDNA. *FEBS Lett* 214 (2), 317 (1987).
50. Darson, M. F., Pacelli, A., Roche, P. et al., Human glandular kallikrein 2 (hK2) expression in prostatic intraepithelial neoplasia and adenocarcinoma: a novel prostate cancer marker. *Urology* 49 (6), 857 (1997).
51. Kumar, A., Mikolajczyk, S. D., Goel, A. S. et al., Expression of pro form of prostate-specific antigen by mammalian cells and its conversion to mature, active form by human kallikrein 2. *Cancer Res* 57 (15), 3111 (1997).
52. Mikolajczyk, S. D., Marker, K. M., Millar, L. S. et al., A truncated precursor form of prostate-specific antigen is a more specific serum marker of prostate cancer. *Cancer Res* 61 (18), 6958 (2001).
53. Peter, J., Unverzagt, C., Krogh, T. N. et al., Identification of precursor forms of free prostate-specific antigen in serum of prostate cancer patients by immunosorption and mass spectrometry. *Cancer Res* 61 (3), 957 (2001).
54. Mikolajczyk, S. D., Millar, L. S., Wang, T. J. et al., A precursor form of prostate-specific antigen is more highly elevated in prostate cancer compared with benign transition zone prostate tissue. *Cancer Res* 60 (3), 756 (2000).
55. Hilz, H., Noldus, J., Hammerer, P. et al., Molecular heterogeneity of free PSA in sera of patients with benign and malignant prostate tumors. *Eur Urol* 36 (4), 286 (1999).
56. Noldus, J., Chen, Z., and Stamey, T. A., Isolation and characterization of free form prostate specific antigen (f-PSA) in sera of men with prostate cancer. *J Urol* 158 (4), 1606 (1997).
57. Mikolajczyk, S. D. and Rittenhouse, H. G., Pro PSA: a more cancer specific form of prostate specific antigen for the early detection of prostate cancer. *Keio J Med* 52 (2), 86 (2003).
58. Mikolajczyk, S. D., Millar, L. S., Wang, T. J. et al., "BPSA," a specific molecular form of free prostate-specific antigen, is found predominantly in the transition zone of patients with nodular benign prostatic hyperplasia. *Urology* 55 (1), 41 (2000).

59. Linton, H. J., Marks, L. S., Millar, L. S. et al., Benign prostate-specific antigen (BPSA) in serum is increased in benign prostate disease. *Clin Chem* 49 (2), 253 (2003).
60. Canto, E. I., Singh, H., Shariat, S. F. et al., Serum BPSA outperforms both total PSA and free PSA as a predictor of prostatic enlargement in men without prostate cancer. *Urology* 63 (5), 905 (2004).
61. Catalona, W. J., Bartsch, G., Rittenhouse, H. G. et al., Serum pro prostate specific antigen improves cancer detection compared to free and complexed prostate specific antigen in men with prostate specific antigen 2 to 4 ng/ml. *J Urol* 170 (6 Pt 1), 2181 (2003).
62. Mikolajczyk, S. D., Catalona, W. J., Evans, C. L. et al., Proenzyme forms of prostate-specific antigen in serum improve the detection of prostate cancer. *Clin Chem* 50 (6), 1017 (2004).
63. Naya, Y., Fritsche, H. A., Bhadkamkar, V. A. et al., Volume-based evaluation of serum assays for new prostate-specific antigen isoforms in the detection of prostate cancer. *Urology* 63 (3), 492 (2004).
64. Naya, Y., Fritsche, H. A., Bhadkamkar, V. A. et al., Evaluation of precursor prostate-specific antigen isoform ratios in the detection of prostate cancer. *Urol Oncol* 23 (1), 16 (2005).
65. Groskopf, J., Aubin, S. M., Deras, I. L. et al., APTIMA PCA3 molecular urine test: development of a method to aid in the diagnosis of prostate cancer. *Clin Chem* 52 (6), 1089 (2006).
66. Haese, A., de la Taille, A., van Poppel, H. et al., Clinical utility of the PCA3 urine assay in European men scheduled for repeat biopsy. *Eur Urol* 54 (5), 1081 (2008).
67. Marks, L. S., Fradet, Y., Deras, I. L. et al., PCA3 molecular urine assay for prostate cancer in men undergoing repeat biopsy. *Urology* 69 (3), 532 (2007).
68. Tinzl, M., Marberger, M., Horvath, S., and Chypre, C., DD3PCA3 RNA analysis in urine--a new perspective for detecting prostate cancer. *Eur Urol* 46 (2), 182 (2004).
69. Roobol, M. J., Schroder, F. H., van Leeuwen, P. et al., Performance of the prostate cancer antigen 3 (PCA3) gene and prostate-specific antigen in prescreened men: exploring the value of PCA3 for a first-line diagnostic test. *Eur Urol* 58 (4), 475 (2010).
70. Tomlins, S. A., Rhodes, D. R., Perner, S. et al., Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. *Science* 310 (5748), 644 (2005).
71. Hessels, D., Smit, F. P., Verhaegh, G. W. et al., Detection of TMPRSS2-ERG fusion transcripts and prostate cancer antigen 3 in urinary sediments may improve diagnosis of prostate cancer. *Clin Cancer Res* 13 (17), 5103 (2007).
72. Aubin SM, Miick S, Williamsen S, Hodge P, Meinke J, Blase A, Hessels D, Schalken J, Rittenhouse H, Groskopf J, Improved prediction of prostate biopsy outcome using PCA3, TMPRSS2:ERG gene fusion and serum PSA. *Journal of Urology Supplement* 179, 725 (2008).

73. Freedland, S. J., Hotaling, J. M., Fitzsimons, N. J. et al., PSA in the new millennium: a powerful predictor of prostate cancer prognosis and radical prostatectomy outcomes--results from the SEARCH database. *Eur Urol* 53 (4), 758 (2008).
74. Chun, F. K., Briganti, A., Gallina, A. et al., Prostate-specific antigen improves the ability of clinical stage and biopsy Gleason sum to predict the pathologic stage at radical prostatectomy in the new millennium. *Eur Urol* 52 (4), 1067 (2007).
75. Gleason, D. F. and Mellinger, G. T., Prediction of prognosis for prostatic adenocarcinoma by combined histological grading and clinical staging. *J Urol* 111 (1), 58 (1974).
76. Albertsen, P. C., Hanley, J. A., Gleason, D. F., and Barry, M. J., Competing risk analysis of men aged 55 to 74 years at diagnosis managed conservatively for clinically localized prostate cancer. *JAMA* 280 (11), 975 (1998).
77. Allsbrook, W. C., Jr., Mangold, K. A., Johnson, M. H. et al., Interobserver reproducibility of Gleason grading of prostatic carcinoma: general pathologist. *Hum Pathol* 32 (1), 81 (2001).
78. Melia, J., Moseley, R., Ball, R. Y. et al., A UK-based investigation of inter- and intra-observer reproducibility of Gleason grading of prostatic biopsies. *Histopathology* 48 (6), 644 (2006).
79. Orozco, R., O'Dowd, G., Kunnel, B. et al., Observations on pathology trends in 62,537 prostate biopsies obtained from urology private practices in the United States. *Urology* 51 (2), 186 (1998).
80. Sakr, W. A., Tefilli, M. V., Grignon, D. J. et al., Gleason score 7 prostate cancer: a heterogeneous entity? Correlation with pathologic parameters and disease-free survival. *Urology* 56 (5), 730 (2000).
81. Catalona, W. J., Bartsch, G., Rittenhouse, H. G. et al., Serum pro-prostate specific antigen preferentially detects aggressive prostate cancers in men with 2 to 4 ng/ml prostate specific antigen. *J Urol* 171 (6 Pt 1), 2239 (2004).
82. de Vries, S. H., Raaijmakers, R., Blijenberg, B. G. et al., Additional use of [-2] precursor prostate-specific antigen and "benign" PSA at diagnosis in screen-detected prostate cancer. *Urology* 65 (5), 926 (2005).
83. Auprich, M., Bjartell, A., Chun, F. K. et al., Contemporary role of prostate cancer antigen 3 in the management of prostate cancer. *Eur Urol* 60 (5), 1045 (2011).
84. Rice, K. R., Chen, Y., Ali, A. et al., Evaluation of the ETS-related gene mRNA in urine for the detection of prostate cancer. *Clin Cancer Res* 16 (5), 1572 (2010).
85. Salagierski, M. and Schalken, J. A., Molecular diagnosis of prostate cancer: PCA3 and TMPRSS2:ERG gene fusion. *J Urol* 187 (3), 795 (2012).
86. Mikolajczyk, S. D. and Rittenhouse, H. G., Tumor-associated forms of prostate specific antigen improve the discrimination of prostate cancer from benign disease. *Rinsho Byori* 52 (3), 223 (2004).

87. Paul, B., Dhir, R., Landsittel, D. et al., Detection of prostate cancer with a blood-based assay for early prostate cancer antigen. *Cancer Res* 65 (10), 4097 (2005).
88. Reiter, R. E., Gu, Z., Watabe, T. et al., Prostate stem cell antigen: a cell surface marker overexpressed in prostate cancer. *Proc Natl Acad Sci U S A* 95 (4), 1735 (1998).
89. Rubin, M. A., Zhou, M., Dhanasekaran, S. M. et al., alpha-Methylacyl coenzyme A racemase as a tissue biomarker for prostate cancer. *Jama* 287 (13), 1662 (2002).
90. Stephan, C., Jung, K., Lein, M. et al., Molecular forms of prostate-specific antigen and human kallikrein 2 as promising tools for early diagnosis of prostate cancer. *Cancer Epidemiol Biomarkers Prev* 9 (11), 1133 (2000).
91. Ravery, V., The significance of recurrent PSA after radical prostatectomy: benign versus malignant sources. *Semin Urol Oncol* 17 (3), 127 (1999).
92. Cookson, M. S., Aus, G., Burnett, A. L. et al., Variation in the definition of biochemical recurrence in patients treated for localized prostate cancer: the American Urological Association Prostate Guidelines for Localized Prostate Cancer Update Panel report and recommendations for a standard in the reporting of surgical outcomes. *J Urol* 177 (2), 540 (2007).
93. Partin, A. W., Pearson, J. D., Landis, P. K. et al., Evaluation of serum prostate-specific antigen velocity after radical prostatectomy to distinguish local recurrence from distant metastases. *Urology* 43 (5), 649 (1994).
94. Pound, C. R., Partin, A. W., Epstein, J. I., and Walsh, P. C., Prostate-specific antigen after anatomic radical retropubic prostatectomy. Patterns of recurrence and cancer control. *Urol Clin North Am* 24 (2), 395 (1997).
95. Pound, C. R., Partin, A. W., Eisenberger, M. A. et al., Natural history of progression after PSA elevation following radical prostatectomy. *JAMA* 281 (17), 1591 (1999).
96. Crook, J. M., Choan, E., Perry, G. A. et al., Serum prostate-specific antigen profile following radiotherapy for prostate cancer: implications for patterns of failure and definition of cure. *Urology* 51 (4), 566 (1998).
97. Critz, F. A., Levinson, A. K., Williams, W. H. et al., The PSA nadir that indicates potential cure after radiotherapy for prostate cancer. *Urology* 49 (3), 322 (1997).
98. Hanlon, A. L., Diratzouian, H., and Hanks, G. E., Posttreatment prostate-specific antigen nadir highly predictive of distant failure and death from prostate cancer. *Int J Radiat Oncol Biol Phys* 53 (2), 297 (2002).
99. Ray, M. E., Thames, H. D., Levy, L. B. et al., PSA nadir predicts biochemical and distant failures after external beam radiotherapy for prostate cancer: a multi-institutional analysis. *Int J Radiat Oncol Biol Phys* 64 (4), 1140 (2006).
100. Roach, M., 3rd, Hanks, G., Thames, H., Jr. et al., Defining biochemical failure following radiotherapy with or without hormonal therapy in men with clinically localized prostate cancer: recommendations of the RTOG-ASTRO Phoenix Consensus Conference. *Int J Radiat Oncol Biol Phys* 65 (4), 965 (2006).

101. Satoh, T., Ishiyama, H., Matsumoto, K. et al., Prostate-specific antigen 'bounce' after permanent 125I-implant brachytherapy in Japanese men: a multi-institutional pooled analysis. *BJU Int* 103 (8), 1064 (2009).
102. Collette, L., Burzykowski, T., and Schroder, F. H., Prostate-specific antigen (PSA) alone is not an appropriate surrogate marker of long-term therapeutic benefit in prostate cancer trials. *Eur J Cancer* 42 (10), 1344 (2006).
103. Verbel, D. A., Heller, G., Kelly, W. K., and Scher, H. I., Quantifying the amount of variation in survival explained by prostate-specific antigen. *Clin Cancer Res* 8 (8), 2576 (2002).
104. Olmos, D., Brewer, D., Clark, J. et al., Prognostic value of blood mRNA expression signatures in castration-resistant prostate cancer: a prospective, two-stage study. *Lancet Oncol* (2012).
105. Ross, R. W., Galsky, M. D., Scher, H. I. et al., A whole-blood RNA transcript-based prognostic model in men with castration-resistant prostate cancer: a prospective study. *Lancet Oncol* (2012).
106. Addona, T. A., Abbatiello, S. E., Schilling, B. et al., Multi-site assessment of the precision and reproducibility of multiple reaction monitoring-based measurements of proteins in plasma. *Nat Biotechnol* 27 (7), 633 (2009).
107. Hortin, G. L., Sviridov, D., and Anderson, N. L., High-abundance polypeptides of the human plasma proteome comprising the top 4 logs of polypeptide abundance. *Clin Chem* 54 (10), 1608 (2008).
108. Ransohoff, D. F., Lessons from controversy: ovarian cancer screening and serum proteomics. *J Natl Cancer Inst* 97 (4), 315 (2005).
109. Kulasingam, V. and Diamandis, E. P., Strategies for discovering novel cancer biomarkers through utilization of emerging technologies. *Nat Clin Pract Oncol* 5 (10), 588 (2008).
110. (CBS), Centraal Bureau voor de Statistiek, Gezondheid, leefsteil, gebruik van zorg, Available at http://statline.cbs.nl/StatWeb/publication/?DM=SLNL&PA=03799&D1=291&D2=0-17&D3=0&D4=a&HDR=G2,T,G3&STB=G1&CHARTTYPE=1&VW=T, (2011).
111. Ishibashi, M., Standardization of prostate-specific antigen (PSA) assays: can interchangeability of PSA measurements be improved? *Clin Chem* 52 (1), 1 (2006).
112. Patel, D., White, P. A., and Milford Ward, A., A comparison of six commercial assays for total and free prostate specific antigen (PSA): the predictive value of the ratio of free to total PSA. *BJU Int* 85 (6), 686 (2000).
113. Semjonow, A., Brandt, B., Oberpenning, F. et al., Discordance of assay methods creates pitfalls for the interpretation of prostate-specific antigen values. *Prostate Suppl* 7, 3 (1996).

114. Yurdakul, G., Bangma, C. H., Blijenberg, B. G. et al., Different PSA assays lead to detection of prostate cancers with identical histological features. *Eur Urol* 42 (2), 154 (2002).
115. Zhou, A. M., Tewari, P. C., Bluestein, B. I. et al., Multiple forms of prostate-specific antigen in serum: differences in immunorecognition by monoclonal and polyclonal assays. *Clin Chem* 39 (12), 2483 (1993).
116. Rafferty, B., Rigsby, P., Rose, M. et al., Reference reagents for prostate-specific antigen (PSA): establishment of the first international standards for free PSA and PSA (90:10). *Clin Chem* 46 (9), 1310 (2000).
117. WHO, WHO Expert Committee on Biological Standardisation. *WHO Report 1999* (1999).
118. Blijenberg, B. G., Yurdakul, G., Van Zelst, B. D. et al., Discordant performance of assays for free and total prostate-specific antigen in relation to the early detection of prostate cancer. *BJU Int* 88 (6), 545 (2001).
119. Kort, S. A., Martens, F., Vanpoucke, H. et al., Comparison of 6 automated assays for total and free prostate-specific antigen with special reference to their reactivity toward the WHO 96/670 reference preparation. *Clin Chem* 52 (8), 1568 (2006).
120. Stamey, T. A., Chen, Z., and Prestigiacomo, A. F., Reference material for PSA: the IFCC standardization study. International Federation of Clinical Chemistry. *Clin Biochem* 31 (6), 475 (1998).
121. de Kok, J. B., Verhaegh, G. W., Roelofs, R. W. et al., DD3(PCA3), a very sensitive and specific marker to detect prostate tumors. *Cancer Res* 62 (9), 2695 (2002).
122. Kumar-Sinha, C., Tomlins, S. A., and Chinnaiyan, A. M., Recurrent gene fusions in prostate cancer. *Nat Rev Cancer* 8 (7), 497 (2008).
123. van Weerden, W. M., de Ridder, C. M., Verdaasdonk, C. L. et al., Development of seven new human prostate tumor xenograft models and their histopathological characterization. *Am J Pathol* 149 (3), 1055 (1996).
124. van den Bemd, G. J., Krijgsveld, J., Luider, T. M. et al., Mass spectrometric identification of human prostate cancer-derived proteins in serum of xenograft-bearing mice. *Mol Cell Proteomics* (2006).
125. Omenn, G. S., States, D. J., Adamski, M. et al., Overview of the HUPO Plasma Proteome Project: results from the pilot phase with 35 collaborating laboratories and multiple analytical groups, generating a core dataset of 3020 proteins and a publicly-available database. *Proteomics* 5 (13), 3226 (2005).
126. Thery, C., Zitvogel, L., and Amigorena, S., Exosomes: composition, biogenesis and function. *Nat Rev Immunol* 2 (8), 569 (2002).
127. Valenti, R., Huber, V., Iero, M. et al., Tumor-released microvesicles as vehicles of immunosuppression. *Cancer Res* 67 (7), 2912 (2007).

128. Valadi, H., Ekstrom, K., Bossios, A. et al., Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol* 9 (6), 654 (2007).
129. Marques, R. B., van Weerden, W. M., Erkens-Schulze, S. et al., The human PC346 xenograft and cell line panel: a model system for prostate cancer progression. *Eur Urol* 49 (2), 245 (2006).
130. Keller, A., Nesvizhskii, A. I., Kolker, E., and Aebersold, R., Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. *Anal Chem* 74 (20), 5383 (2002).
131. Nesvizhskii, A. I., Keller, A., Kolker, E., and Aebersold, R., A statistical model for identifying proteins by tandem mass spectrometry. *Anal Chem* 75 (17), 4646 (2003).
132. Baumeister, W., Walz, J., Zuhl, F., and Seemuller, E., The proteasome: paradigm of a self-compartmentalizing protease. *Cell* 92 (3), 367 (1998).
133. Elsasser, S., Schmidt, M., and Finley, D., Characterization of the proteasome using native gel electrophoresis. *Methods Enzymol* 398, 353 (2005).
134. Dutaud, D., Aubry, L., Henry, L. et al., Development and evaluation of a sandwich ELISA for quantification of the 20S proteasome in human plasma. *J Immunol Methods* 260 (1-2), 183 (2002).
135. Hegmans, J. P., Bard, M. P., Hemmes, A. et al., Proteomic analysis of exosomes secreted by human mesothelioma cells. *Am J Pathol* 164 (5), 1807 (2004).
136. Mortz, E., Krogh, T. N., Vorum, H., and Gorg, A., Improved silver staining protocols for high sensitivity protein identification using matrix-assisted laser desorption/ ionization-time of flight analysis. *Proteomics* 1 (11), 1359 (2001).
137. Claverol, S., Burlet-Schiltz, O., Girbal-Neuhauser, E. et al., Mapping and structural dissection of human 20 S proteasome using proteomic approaches. *Mol Cell Proteomics* 1 (8), 567 (2002).
138. Hermans, K. G., van Marion, R., van Dekken, H. et al., TMPRSS2:ERG fusion by translocation or interstitial deletion is highly relevant in androgen-dependent prostate cancer, but is bypassed in late-stage androgen receptor-negative prostate cancer. *Cancer Res* 66 (22), 10658 (2006).
139. Kumatori, A., Tanaka, K., Inamura, N. et al., Abnormally high expression of proteasomes in human leukemic cells. *Proc Natl Acad Sci U S A* 87 (18), 7071 (1990).
140. Milano, A., Iaffaioli, R. V., and Caponigro, F., The proteasome: a worthwhile target for the treatment of solid tumours? *Eur J Cancer* 43 (7), 1125 (2007).
141. Byrne, J. C., Downes, M. R., O'Donoghue, N. et al., 2D-DIGE as a strategy to identify serum markers for the progression of prostate cancer. *J Proteome Res* 8 (2), 942 (2009).
142. Kanayama, H., Tanaka, K., Aki, M. et al., Changes in expressions of proteasome and ubiquitin genes in human renal cancer cells. *Cancer Res* 51 (24), 6677 (1991).

143. Voorhees, P. M., Dees, E. C., O'Neil, B., and Orlowski, R. Z., The proteasome as a target for cancer therapy. *Clin Cancer Res* 9 (17), 6316 (2003).
144. Wada, M., Kosaka, M., Saito, S. et al., Serum concentration and localization in tumor cells of proteasomes in patients with hematologic malignancy and their pathophysiologic significance. *J Lab Clin Med* 121 (2), 215 (1993).
145. Ma, W., Kantarjian, H., O'Brien, S. et al., Enzymatic activity of circulating proteasomes correlates with clinical behavior in patients with chronic lymphocytic leukemia. *Cancer* (2008).
146. Zoeger, A., Blau, M., Egerer, K. et al., Circulating proteasomes are functional and have a subtype pattern distinct from 20S proteasomes in major blood cells. *Clin Chem* 52 (11), 2079 (2006).
147. Almeida, C. G., Takahashi, R. H., and Gouras, G. K., Beta-amyloid accumulation impairs multivesicular body sorting by inhibiting the ubiquitin-proteasome system. *J Neurosci* 26 (16), 4277 (2006).
148. Dong, J., Chen, W., Welford, A., and Wandinger-Ness, A., The proteasome alpha-subunit XAPC7 interacts specifically with Rab7 and late endosomes. *J Biol Chem* 279 (20), 21334 (2004).
149. Utleg, A. G., Yi, E. C., Xie, T. et al., Proteomic analysis of human prostasomes. *Prostate* 56 (2), 150 (2003).
150. Caby, M. P., Lankar, D., Vincendeau-Scherrer, C. et al., Exosomal-like vesicles are present in human blood plasma. *Int Immunol* 17 (7), 879 (2005).
151. Stoeck, A., Keller, S., Riedle, S. et al., A role for exosomes in the constitutive and stimulus-induced ectodomain cleavage of L1 and CD44. *Biochem J* 393 (Pt 3), 609 (2006).
152. Hao, S., Ye, Z., Li, F. et al., Epigenetic transfer of metastatic activity by uptake of highly metastatic B16 melanoma cell-released exosomes. *Exp Oncol* 28 (2), 126 (2006).
153. Ginestra, A., La Placa, M. D., Saladino, F. et al., The amount and proteolytic content of vesicles shed by human cancer cell lines correlates with their in vitro invasiveness. *Anticancer Res* 18 (5A), 3433 (1998).
154. Hashida, H., Takabayashi, A., Tokuhara, T. et al., Clinical significance of transmembrane 4 superfamily in colon cancer. *Br J Cancer* 89 (1), 158 (2003).
155. Kuefer, R., Day, K. C., Kleer, C. G. et al., ADAM15 disintegrin is associated with aggressive prostate and breast cancer disease. *Neoplasia* 8 (4), 319 (2006).
156. Le, L., Chi, K., Tyldesley, S. et al., Identification of serum amyloid A as a biomarker to distinguish prostate cancer patients with bone lesions. *Clin Chem* 51 (4), 695 (2005).
157. Malik, G., Ward, M. D., Gupta, S. K. et al., Serum levels of an isoform of apolipoprotein A-II as a potential marker for prostate cancer. *Clin Cancer Res* 11 (3), 1073 (2005).
158. Villanueva, J., Shaffer, D. R., Philip, J. et al., Differential exoprotease activities confer tumor-specific serum peptidome patterns. *J Clin Invest* 116 (1), 271 (2006).

159. Lam, Y. W., Mobley, J. A., Evans, J. E. et al., Mass profiling-directed isolation and identification of a stage-specific serologic protein biomarker of advanced prostate cancer. *Proteomics* 5 (11), 2927 (2005).
160. Orazine, C. I., Hincapie, M., Hancock, W. S. et al., A proteomic analysis of the plasma glycoproteins of a MCF-7 mouse xenograft: a model system for the detection of tumor markers. *J Proteome Res* 7 (4), 1542 (2008).
161. Faca, V. M., Song, K. S., Wang, H. et al., A mouse to human search for plasma proteome changes associated with pancreatic tumor development. *PLoS Med* 5 (6), e123 (2008).
162. Grant, A. G. and Duke, D., Production of antibodies against antigens released from human pancreatic tumour xenografts. *Br J Cancer* 44 (3), 388 (1981).
163. Horoszewicz, J. S., Leong, S. S., Kawinski, E. et al., LNCaP model of human prostatic carcinoma. *Cancer Res* 43 (4), 1809 (1983).
164. Schroder, F. H. and Bangma, C. H., The European Randomized Study of Screening for Prostate Cancer (ERSPC). *Br J Urol* 79 Suppl 1, 68 (1997).
165. Lin, J. F., Xu, J., Tian, H. Y. et al., Identification of candidate prostate cancer biomarkers in prostate needle biopsy specimens using proteomic analysis. *Int J Cancer* 121 (12), 2596 (2007).
166. Ben Aicha, S., Lessard, J., Pelletier, M. et al., Transcriptional profiling of genes that are regulated by the endoplasmic reticulum-bound transcription factor AIbZIP/CREB3L4 in prostate cells. *Physiol Genomics* 31 (2), 295 (2007).
167. Ricote, M., Garcia-Tunon, I., Bethencourt, F. et al., The p38 transduction pathway in prostatic neoplasia. *J Pathol* 208 (3), 401 (2006).
168. Cho-Vega, J. H., Tsavachidis, S., Do, K. A. et al., Dicarbonyl/L-xylulose reductase: a potential biomarker identified by laser-capture microdissection-micro serial analysis of gene expression of human prostate adenocarcinoma. *Cancer Epidemiol Biomarkers Prev* 16 (12), 2615 (2007).
169. Mavis, C. K., Morey Kinney, S. R., Foster, B. A., and Karpf, A. R., Expression level and DNA methylation status of glutathione-S-transferase genes in normal murine prostate and TRAMP tumors. *Prostate* 69 (12), 1312 (2009).
170. Meyer-Siegler, K. L., Iczkowski, K. A., Leng, L. et al., Inhibition of macrophage migration inhibitory factor or its receptor (CD74) attenuates growth and invasion of DU-145 prostate cancer cells. *J Immunol* 177 (12), 8730 (2006).
171. Wu, Y. M., Robinson, D. R., and Kung, H. J., Signal pathways in up-regulation of chemokines by tyrosine kinase MER/NYK in prostate cancer cells. *Cancer Res* 64 (20), 7311 (2004).
172. Stewart, J. R. and O'Brian, C. A., Protein kinase C-{alpha} mediates epidermal growth factor receptor transactivation in human prostate cancer cells. *Mol Cancer Ther* 4 (5), 726 (2005).

173. O'Kane, H. F., Watson, C. J., Johnston, S. R. et al., Targeting death receptors in bladder, prostate and renal cancer. *J Urol* 175 (2), 432 (2006).
174. Kwon, E. M., Salinas, C. A., Kolb, S. et al., Genetic polymorphisms in inflammation pathway genes and prostate cancer risk. *Cancer Epidemiol Biomarkers Prev* 20 (5), 923 (2011).
175. Matarasso, N., Bar-Shira, A., Rozovski, U. et al., Functional analysis of the Aurora Kinase A Ile31 allelic variant in human prostate. *Neoplasia* 9 (9), 707 (2007).
176. Wang, J., Krill, D., Torbenson, M. et al., Expression of cadherins and catenins in paired tumor and non-neoplastic primary prostate cultures and corresponding prostatectomy specimens. *Urol Res* 28 (5), 308 (2000).
177. Aaltomaa, S., Lipponen, P., Viitanen, J. et al., Prognostic value of CD44 standard, variant isoforms 3 and 6 and -catenin expression in local prostate cancer treated by radical prostatectomy. *Eur Urol* 38 (5), 555 (2000).
178. Dong, J. T., Prevalent mutations in prostate cancer. *J Cell Biochem* 97 (3), 433 (2006).
179. Weichert, W., Schmidt, M., Gekeler, V. et al., Polo-like kinase 1 is overexpressed in prostate cancer and linked to higher tumor grades. *Prostate* 60 (3), 240 (2004).
180. Wells, C. M., Whale, A. D., Parsons, M. et al., PAK4: a pluripotent kinase that regulates prostate cancer cell adhesion. *J Cell Sci* 123 (Pt 10), 1663 (2010).
181. Untergasser, G., Gander, R., Lilg, C. et al., Profiling molecular targets of TGF-beta1 in prostate fibroblast-to-myofibroblast transdifferentiation. *Mech Ageing Dev* 126 (1), 59 (2005).
182. Joshi, B. H., Leland, P., Calvo, A. et al., Human adrenomedullin up-regulates interleukin-13 receptor alpha2 chain in prostate cancer in vitro and in vivo: a novel approach to sensitize prostate cancer to anticancer therapy. *Cancer Res* 68 (22), 9311 (2008).
183. Wang, Y., Shao, C., Shi, C. H. et al., Change of the cell cycle after flutamide treatment in prostate cancer cells and its molecular mechanism. *Asian J Androl* 7 (4), 375 (2005).
184. Bull, J. H., Ellison, G., Patel, A. et al., Identification of potential diagnostic markers of prostate cancer and prostatic intraepithelial neoplasia using cDNA microarray. *Br J Cancer* 84 (11), 1512 (2001).
185. Oudes, A. J., Roach, J. C., Walashek, L. S. et al., Application of Affymetrix array and Massively Parallel Signature Sequencing for identification of genes involved in prostate cancer progression. *BMC Cancer* 5, 86 (2005).
186. Lutchman, M., Pack, S., Kim, A. C. et al., Loss of heterozygosity on 8p in prostate cancer implicates a role for dematin in tumor progression. *Cancer Genet Cytogenet* 115 (1), 65 (1999).
187. Kniazev Iu, P., Cheburkin Iu, V., Spikermann, K. et al., [Gene expression profiles of protein kinases and phosphatases obtained by hybridization with cDNA arrays: molecular portrait of human prostate carcinoma]. *Mol Biol (Mosk)* 37 (1), 97 (2003).

188. Simak, R., Capodieci, P., Cohen, D. W. et al., Expression of c-kit and kit-ligand in benign and malignant prostatic tissues. *Histol Histopathol* 15 (2), 365 (2000).
189. Di Lorenzo, G., Autorino, R., D'Armiento, F. P. et al., Expression of proto-oncogene c-kit in high risk prostate cancer. *Eur J Surg Oncol* 30 (9), 987 (2004).
190. Tycko, B., Li, C. M., and Buttyan, R., The Wnt/beta-catenin pathway in Wilms tumors and prostate cancers. *Curr Mol Med* 7 (5), 479 (2007).
191. Bartholow, T. L., Chandran, U. R., Becich, M. J., and Parwani, A. V., Immunohistochemical staining of radixin and moesin in prostatic adenocarcinoma. *BMC Clin Pathol* 11, 1 (2011).
192. Adams, J., The proteasome: structure, function, and role in the cell. *Cancer Treat Rev* 29 Suppl 1, 3 (2003).
193. Danilova, N. P., Mertts, M. V., Sorokolit, T. P., and Vasilov, R. G., [Individual variation of immune response to thyroid hormones in BALB/c mice]. *Biull Eksp Biol Med* 119 (1), 80 (1995).
194. McKean, D., Huppi, K., Bell, M. et al., Generation of antibody diversity in the immune response of BALB/c mice to influenza virus hemagglutinin. *Proc Natl Acad Sci U S A* 81 (10), 3180 (1984).
195. Harper, R. G., Workman, S. R., Schuetzner, S. et al., Low-molecular-weight human serum proteome using ultrafiltration, isoelectric focusing, and mass spectrometry. *Electrophoresis* 25 (9), 1299 (2004).
196. Tirumalai, R. S., Chan, K. C., Prieto, D. A. et al., Characterization of the low molecular weight human serum proteome. *Mol Cell Proteomics* 2 (10), 1096 (2003).
197. Jacob, A. N., Kalapurakal, J., Davidson, W. R. et al., A receptor tyrosine kinase, UFO/Axl, and other genes isolated by a modified differential display PCR are overexpressed in metastatic prostatic carcinoma cell line DU145. *Cancer Detect Prev* 23 (4), 325 (1999).
198. Mahajan, N. P., Whang, Y. E., Mohler, J. L., and Earp, H. S., Activated tyrosine kinase Ack1 promotes prostate tumorigenesis: role of Ack1 in polyubiquitination of tumor suppressor Wwox. *Cancer Res* 65 (22), 10514 (2005).
199. Sainaghi, P. P., Castello, L., Bergamasco, L. et al., Gas6 induces proliferation in prostate carcinoma cell lines expressing the Axl receptor. *J Cell Physiol* 204 (1), 36 (2005).
200. Rhodes, D. R., Yu, J., Shanker, K. et al., ONCOMINE: a cancer microarray database and integrated data-mining platform. *Neoplasia* 6 (1), 1 (2004).
201. Mikolajczyk, S. D., Millar, L. S., Marker, K. M. et al., Seminal plasma contains "BPSA," a molecular form of prostate-specific antigen that is associated with benign prostatic hyperplasia. *Prostate* 45 (3), 271 (2000).

202. Sokoll, L. J., Wang, Y., Feng, Z. et al., [-2]proenzyme prostate specific antigen for prostate cancer detection: a national cancer institute early detection research network validation study. *J Urol* 180 (2), 539 (2008).
203. Stephan, C., Kahrs, A. M., Cammann, H. et al., A [-2]proPSA-based artificial neural network significantly improves differentiation between prostate cancer and benign prostatic diseases. *Prostate* 69 (2), 198 (2009).
204. Stephan, C., Cammann, H., Deger, S. et al., Benign prostatic hyperplasia-associated free prostate-specific antigen improves detection of prostate cancer in an artificial neural network. *Urology* 74 (4), 873 (2009).
205. Schroder, F. H., Hugosson, J., Roobol, M. J. et al., Screening and prostate-cancer mortality in a randomized European study. *N Engl J Med* 360 (13), 1320 (2009).
206. Berger, A. P., Deibl, M., Steiner, H. et al., Longitudinal PSA changes in men with and without prostate cancer: assessment of prostate cancer risk. *Prostate* 64 (3), 240 (2005).
207. DeLong, E. R., DeLong, D. M., and Clarke-Pearson, D. L., Comparing the areas under two or more correlated receiver operating characteristic curves: a nonparametric approach. *Biometrics* 44 (3), 837 (1988).
208. Hugosson, J., Aus, G., Lilja, H. et al., Results of a randomized, population-based study of biennial screening using serum prostate-specific antigen measurement to detect prostate carcinoma. *Cancer* 100 (7), 1397 (2004).
209. Postma, R., Schroder, F. H., van Leenders, G. J. et al., Cancer detection and cancer characteristics in the European Randomized Study of Screening for Prostate Cancer (ERSPC)--Section Rotterdam. A comparison of two rounds of screening. *Eur Urol* 52 (1), 89 (2007).
210. van der Cruijsen-Koeter, I. W., Roobol, M. J., Wildhagen, M. F. et al., Tumor characteristics and prognostic factors in two subsequent screening rounds with four-year interval within prostate cancer screening trial, ERSPC Rotterdam. *Urology* 68 (3), 615 (2006).
211. Oberpenning, F., Weining, C., Brandt, B. et al., A new modification of the Chiron ACS assay for total prostate-specific antigen achieves equimolar response characteristics and improves the detection of prostate cancer. *Clin Chem Lab Med* 41 (1), 90 (2003).
212. Semjonow, A., Oberpenning, F., Weining, C. et al., Do modifications of nonequimolar assays for total prostate-specific antigen improve detection of prostate cancer? *Clin Chem* 47 (8), 1472 (2001).
213. D'Amico, A. V., Chen, M. H., Roehl, K. A., and Catalona, W. J., Preoperative PSA velocity and the risk of death from prostate cancer after radical prostatectomy. *N Engl J Med* 351 (2), 125 (2004).
214. D'Amico, A. V., Kantoff, P., Loffredo, M. et al., Predictors of mortality after prostate-specific antigen failure. *Int J Radiat Oncol Biol Phys* 65 (3), 656 (2006).

215. D'Amico, A. V., Renshaw, A. A., Sussman, B., and Chen, M. H., Pretreatment PSA velocity and risk of death from prostate cancer following external beam radiation therapy. *Jama* 294 (4), 440 (2005).
216. Link, R. E., Shariat, S. F., Nguyen, C. V. et al., Variation in prostate specific antigen results from 2 different assay platforms: clinical impact on 2304 patients undergoing prostate cancer screening. *J Urol* 171 (6 Pt 1), 2234 (2004).
217. Sengupta, S., Myers, R. P., Slezak, J. M. et al., Preoperative prostate specific antigen doubling time and velocity are strong and independent predictors of outcomes following radical prostatectomy. *J Urol* 174 (6), 2191 (2005).
218. Stephan, C., Klaas, M., Muller, C. et al., Interchangeability of measurements of total and free prostate-specific antigen in serum with 5 frequently used assay combinations: an update. *Clin Chem* 52 (1), 59 (2006).
219. Stephan, C. and Semjonow, A., Accuracy assessment of PSA methods must be based on clinical instead of artificial samples--a response to AW Roddam et al., Ann Clin Biochem 2006; 43: 35-48. *Ann Clin Biochem* 43 (Pt 5), 421 (2006).
220. Zelst, BD and Blijenberg, BG, PSA-comparability: standardization or harmonization? *Ned Tijdschr Klin Chem* 27, 25 (2002).
221. Roddam, A. W., Rimmer, J., Nickerson, C., and Ward, A. M., Prostate-specific antigen: bias and molarity of commercial assays for PSA in use in England. *Ann Clin Biochem* 43 (Pt 1), 35 (2006).
222. Catalona, W. J., Richie, J. P., Ahmann, F. R. et al., Comparison of digital rectal examination and serum prostate specific antigen in the early detection of prostate cancer: results of a multicenter clinical trial of 6,630 men. *J Urol* 151 (5), 1283 (1994).
223. Roobol, M. J., Kirkels, W. J., and Schroder, F. H., Features and preliminary results of the Dutch centre of the ERSPC (Rotterdam, the Netherlands). *BJU Int* 92 Suppl 2, 48 (2003).
224. Roobol, M. J. and Schroder, F. H., European Randomized Study of Screening for Prostate Cancer: achievements and presentation. *BJU Int* 92 Suppl 2, 117 (2003).
225. Rowlands, R. J., Griffiths, K., Kemp, K. W. et al., Application of Cusum techniques to the routine monitoring of analytical performance in clinical laboratories. *Stat Med* 2 (2), 141 (1983).
226. Raaijmakers, R., Blijenberg, B. G., Finlay, J. A. et al., Prostate cancer detection in the prostate specific antigen range of 2.0 to 3.9 ng/ml: value of percent free prostate specific antigen on tumor detection and tumor aggressiveness. *J Urol* 171 (6 Pt 1), 2245 (2004).
227. Roobol, M. J., Kranse, R., de Koning, H. J., and Schroder, F. H., Prostate-specific antigen velocity at low prostate-specific antigen levels as screening tool for prostate cancer: results of second screening round of ERSPC (ROTTERDAM). *Urology* 63 (2), 309 (2004).

228. Thompson, I. M., Ankerst, D. P., Chi, C. et al., Assessing prostate cancer risk: results from the Prostate Cancer Prevention Trial. *J Natl Cancer Inst* 98 (8), 529 (2006).
229. Roddam, A. W., Price, C. P., Allen, N. E., and Ward, A. M., Assessing the clinical impact of prostate-specific antigen assay variability and nonequimolarity: a simulation study based on the population of the United Kingdom. *Clin Chem* 50 (6), 1012 (2004).
230. Sotelo, R. J., Mora, K. E., Perez, L. H. et al., Assay standardization bias: different prostate cancer detection rates and clinical outcomes resulting from different assays for free and total prostate-specific antigen. *Urology* 69 (6), 1143 (2007).
231. Draisma, G., Boer, R., Otto, S. J. et al., Lead times and overdetection due to prostate-specific antigen screening: estimates from the European Randomized Study of Screening for Prostate Cancer. *J Natl Cancer Inst* 95 (12), 868 (2003).
232. Schroder, F. H., Bangma, C. H., and Roobol, M. J., Is It Necessary to Detect All Prostate Cancers in Men with Serum PSA Levels <3.0ng/ml? A Comparison of Biopsy Results of PCPT and Outcome-Related Information from ERSPC. *Eur Urol* (2008).
233. Schroder, F. H., Carter, H. B., Wolters, T. et al., Early Detection of Prostate Cancer in 2007 Part 1: PSA and PSA Kinetics. *Eur Urol* 53 (3), 468 (2008).
234. Steyerberg, E. W., Roobol, M. J., Kattan, M. W. et al., Prediction of indolent prostate cancer: validation and updating of a prognostic nomogram. *J Urol* 177 (1), 107 (2007).
235. Candas, B., Cusan, L., Gomez, J. L. et al., Evaluation of prostatic specific antigen and digital rectal examination as screening tests for prostate cancer. *Prostate* 45 (1), 19 (2000).
236. Gosselaar, C., Roobol, M. J., Roemeling, S. et al., Screening for prostate cancer without digital rectal examination and transrectal ultrasound: results after four years in the European Randomized Study of Screening for Prostate Cancer (ERSPC), Rotterdam. *Prostate* 66 (6), 625 (2006).
237. Gosselaar, C., Roobol, M. J., Roemeling, S. et al., Screening for prostate cancer at low PSA range: the impact of digital rectal examination on tumor incidence and tumor characteristics. *Prostate* 67 (2), 154 (2007).
238. Schroder, F. H., Roobol-Bouts, M., Vis, A. N. et al., Prostate-specific antigen-based early detection of prostate cancer--validation of screening without rectal examination. *Urology* 57 (1), 83 (2001).
239. Schroder, F. H., van der Maas, P., Beemsterboer, P. et al., Evaluation of the digital rectal examination as a screening test for prostate cancer. Rotterdam section of the European Randomized Study of Screening for Prostate Cancer. *J Natl Cancer Inst* 90 (23), 1817 (1998).
240. Diamandis, E. P., The failure of protein cancer biomarkers to reach the clinic: why, and what can be done to address the problem? *BMC Med* 10, 87 (2012).

241. de Martino, M., Hoetzenecker, K., Ankersmit, H. J. et al., Serum 20S proteasome is elevated in patients with renal cell carcinoma and associated with poor prognosis. *Br J Cancer* 106 (5), 904 (2012).
242. Hoffmann, O., Heubner, M., Anlasik, T. et al., Circulating 20S proteasome in patients with non-metastasized breast cancer. *Anticancer Res* 31 (6), 2197 (2012).
243. O'Hurley, G., O'Grady, A., Smyth, P. et al., Evaluation of Zinc-alpha-2-Glycoprotein and Proteasome Subunit beta-Type 6 Expression in Prostate Cancer Using Tissue Microarray Technology. *Appl Immunohistochem Mol Morphol* (2010).
244. Romanuik, T. L., Ueda, T., Le, N. et al., Novel biomarkers for prostate cancer including noncoding transcripts. *Am J Pathol* 175 (6), 2264 (2009).
245. Yamada, Y., Tomaru, U., Ishizu, A. et al., Expression of proteasome subunit beta5t in thymic epithelial tumors. *Am J Surg Pathol* 35 (9), 1296 (2011).
246. Battke, C., Ruiss, R., Welsch, U. et al., Tumour exosomes inhibit binding of tumour-reactive antibodies to tumour cells and reduce ADCC. *Cancer Immunol Immunother* (2011).
247. Gyorgy, B., Szabo, T. G., Pasztoi, M. et al., Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. *Cell Mol Life Sci* 68 (16), 2667 (2011).
248. Lai, R. C., Tan, S. S., Teh, B. J. et al., Proteolytic Potential of the MSC Exosome Proteome: Implications for an Exosome-Mediated Delivery of Therapeutic Proteasome. *Int J Proteomics* 2012, 971907 (2012).
249. Nilsson, J., Skog, J., Nordstrand, A. et al., Prostate cancer-derived urine exosomes: a novel approach to biomarkers for prostate cancer. *Br J Cancer* 100 (10), 1603 (2009).
250. Ekström, K; Valadi, H; Sjöstrand, M; Malmhäll, C; Bossios, A; Eldh, M; Lötvall, J, Characterization of mRNA and microRNA in human mast cell-derived exosomes and their transfer to other mast cells and blood CD34 progenitor cells. *Journal of Extracellular Vesicles 2012* 1, http://dx.doi.org/10.3402/jev.v1i0.18389 (2012).
251. Skog, J., Wurdinger, T., van Rijn, S. et al., Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat Cell Biol* 10 (12), 1470 (2008).
252. Logozzi, M., De Milito, A., Lugini, L. et al., High levels of exosomes expressing CD63 and caveolin-1 in plasma of melanoma patients. *PLoS One* 4 (4), e5219 (2009).
253. Silva, J., Garcia, V., Rodriguez, M. et al., Analysis of exosome release and its prognostic value in human colorectal cancer. *Genes Chromosomes Cancer* 51 (4), 409 (2012).
254. Zhou, H., Yuen, P. S., Pisitkun, T. et al., Collection, storage, preservation, and normalization of human urinary exosomes for biomarker discovery. *Kidney Int* 69 (8), 1471 (2006).
255. Bryant, R. J., Pawlowski, T., Catto, J. W. et al., Changes in circulating microRNA levels associated with prostate cancer. *Br J Cancer* 106 (4), 768 (2012).

256. Khan, S., Jutzy, J. M., Valenzuela, M. M. et al., Plasma-derived exosomal survivin, a plausible biomarker for early detection of prostate cancer. *PLoS One* 7 (10), e46737 (2012).
257. Kuslich, C; Pawlowski, T.L.; Deng, T; Tinder, T; Kim, J; Kimbrough, J; Spetzler, D;, A sensitive exosome-based biosignature for the diagnosis of prostate cancer. *Journal of Clinical Oncology* 28 (15s), 2010 (2010).
258. Mitchell, P. J., Welton, J., Staffurth, J. et al., Can urinary exosomes act as treatment response markers in prostate cancer? *J Transl Med* 7, 4 (2009).
259. Somasundaram, R; Meenhard, H, Melanoma exosomes: messengers of metastasis. *Nature Medicine* 18 (6), 853 (2012).
260. Linger, R. M., Cohen, R. A., Cummings, C. T. et al., Mer or Axl receptor tyrosine kinase inhibition promotes apoptosis, blocks growth and enhances chemosensitivity of human non-small cell lung cancer. *Oncogene* (2012).
261. Paccez, J. D., Vasques, G. J., Correa, R. G. et al., The receptor tyrosine kinase Axl is an essential regulator of prostate cancer proliferation and tumor growth and represents a new therapeutic target. *Oncogene* (2012).
262. Shapiro, G. I., McCallum, S., Adams, L. M. et al., A Phase 1 dose-escalation study of the safety and pharmacokinetics of once-daily oral foretinib, a multi-kinase inhibitor, in patients with solid tumors. *Invest New Drugs* (2012).
263. Bossuyt, P. M., Reitsma, J. B., Bruns, D. E. et al., The STARD statement for reporting studies of diagnostic accuracy: explanation and elaboration. *Clin Chem* 49 (1), 7 (2003).
264. McShane, L. M., Altman, D. G., Sauerbrei, W. et al., REporting recommendations for tumor MARKer prognostic studies (REMARK). *Breast Cancer Res Treat* 100 (2), 229 (2006).
265. Catalona, W. J., Partin, A. W., Sanda, M. G. et al., A multicenter study of [-2]pro-prostate specific antigen combined with prostate specific antigen and free prostate specific antigen for prostate cancer detection in the 2.0 to 10.0 ng/ml prostate specific antigen range. *J Urol* 185 (5), 1650 (2011).
266. Guazzoni, G., Nava, L., Lazzeri, M. et al., Prostate-specific antigen (PSA) isoform p2PSA significantly improves the prediction of prostate cancer at initial extended prostate biopsies in patients with total PSA between 2.0 and 10 ng/ml: results of a prospective study in a clinical setting. *Eur Urol* 60 (2), 214 (2011).
267. Lazzeri, M., Haese, A., de la Taille, A. et al., Serum isoform [-2]proPSA derivatives significantly improve prediction of prostate cancer at initial biopsy in a total PSA range of 2-10 ng/ml: a multicentric European study. *Eur Urol* 63 (6), 986 (2013).
268. Le, B. V., Griffin, C. R., Loeb, S. et al., [-2]Proenzyme prostate specific antigen is more accurate than total and free prostate specific antigen in differentiating prostate cancer from benign disease in a prospective prostate cancer screening study. *J Urol* 183 (4), 1355 (2010).

269. Stephan, C., Vincendeau, S., Houlgatte, A. et al., Multicenter evaluation of [-2] proprostate-specific antigen and the prostate health index for detecting prostate cancer. *Clin Chem* 59 (1), 306 (2013).
270. Filella, X. and Gimenez, N., Evaluation of [-2] proPSA and Prostate Health Index (phi) for the detection of prostate cancer: a systematic review and meta-analysis. *Clin Chem Lab Med* 51 (4), 729 (2013).
271. Perdona, S., Bruzzese, D., Ferro, M. et al., Prostate health index (phi) and prostate cancer antigen 3 (PCA3) significantly improve diagnostic accuracy in patients undergoing prostate biopsy. *Prostate* 73 (3), 227 (2013).
272. Scattoni, V., Lazzeri, M., Lughezzani, G. et al., Head-to-Head Comparison of Prostate Health Index and Urinary PCA3 for Predicting Cancer at Initial or Repeat Biopsy. *J Urol* (2013).
273. Stephan, C., Jung, K., Semjonow, A. et al., Comparative assessment of urinary prostate cancer antigen 3 and TMPRSS2:ERG gene fusion with the serum [-2]proprostate-specific antigen-based prostate health index for detection of prostate cancer. *Clin Chem* 59 (1), 280 (2013).
274. Nichol, M. B., Wu, J., An, J. J. et al., Budget impact analysis of a new prostate cancer risk index for prostate cancer detection. *Prostate Cancer Prostatic Dis* 14 (3), 253 (2011).
275. Fillee, C., Tombal, B., and Philippe, M., Prostate cancer screening: clinical impact of WHO calibration of Beckman Coulter Access prostate-specific antigen assays. *Clin Chem Lab Med* 48 (2), 285 (2010).

PHD PORTFOLIO

Summary of PhD training and teaching

ERASMUS UNIVERSITEIT ROTTERDAM

Name PhD student: Flip H. Jansen Erasmus MC Department: Urology	PhD period: 2005-2008 Promotor: prof.dr. G. Jenster

1. PhD training		
	Year	**Workload (ECTS)**
Specific courses (e.g. Research school, Medical Training) – Morello Web Editor Course	2008	0.5
Seminars and workshops – Department's Journal Club – Department's 'refereeravond' – Department's 'promovendiavond'	2005-2008 2005-2008 2005-2008	1 1 1
Presentations – Multiple presentations at different conventions and meetings	2005-2008	5
(Inter)national conferences – Multiple poster and oral presentations at EAU, AUA, AACR, ESUR and IFCC-EFLM meetings	2005-2008	20
Other – Clinical work urological ward	2008	2

2. Teaching		
	Year	**Workload (Hours/ ECTS)**
Lecturing – Medical students of Erasmus MC	2008	1

EAU	European Association of Urology
AUA	American Urological Association
AACR	American Association for Cancer Research
ESUR	EAU Section of Urological Research
IFCC-EFLM	European Congress of Clinical Chemistry and Laboratory Medicine

ABOUT THE AUTHOR

Flip Jansen was born on February 17th 1980 in Gouda. He soon moved to Zeist where he followed primary and secondary education. After graduating from the gymnasium in 1998 he moved to Utrecht and started his medical education at Utrecht University. During his surgical internship at St. Jansdal Hospital in Harderwijk he became enthusiastic about the field of Urology. His interest in medical research started during a research internship at the department of Haematology under the supervision of dr. R. Fijnheer and professor Ph.G. de Groot. After his graduation in June 2005 he started his PhD research on prostate cancer biomarkers at the Erasmus MC under the guidance of professor Chris Bangma and professor Guido Jenster. In 2008 he applied for the Urological traineeship and in January 2009 he started his two-year residency in Surgery at the Diakonessen Hospital in Utrecht, under supervision of dr. G.J. Clevers. He then performed the first part of his residency in Urology at the Jeroen Bosch Hospital in 's-Hertogenbosch under the supervision of dr. H.P. Beerlage. From January 2013 he is following the last two years of his residency at the University Medical Center in Utrecht under supervision of professor J.L.H.R. Bosch.