



## Review – Prostate Cancer

# Serum Markers for Prostate Cancer: A Rational Approach to the Literature

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### Article info

#### Article history:

Accepted January 4, 2008  
 Published online ahead of  
 print on January 22, 2008

#### Keywords:

Biomarkers  
 Prostate cancer  
 PSA  
 Screening

### Abstract

**Introduction:** Due to its universal applicability for early detection and prediction of cancer stage and disease recurrence, widespread implementation of serum-based prostate-specific antigen (PSA) measurements has a significant influence on current treatment strategies for men with prostate cancer (PCa). However, over-detection and the resultant over-treatment of indolent cancers have been strongly implicated to occur. Using current recommended guidelines, the PSA test suffers from both limited sensitivity and specificity to enable efficacious population-based cancer detection. Therefore, novel biomarkers are much needed to complement PSA by enhancing its diagnostic and prognostic performance.

**Methods:** The present literature on serum markers for PCa was reviewed. PSA derivatives, molecular PSA isoforms, and novel molecular targets in blood were summarized and weighted against their potential to improve decision-making of men with PCa.

**Results:** Current evidence suggests that no single analyte is likely to achieve the desired level of diagnostic and prognostic accuracy for PCa. However, the combination of biomarkers with clinical and demographic data, for example, using established standard nomograms, has produced progress toward the goal of both optimal screening and risk assessment. Furthermore, potential candidate molecular markers for PCa can be derived from high-throughput technologies. Current studies demonstrate that understanding dynamic PSA changes over time may offer diagnostic and prognostic information.

**Conclusions:** Bridging the gap between basic science and clinical practice represents the main goal in the near future to enable physicians to tailor risk-adjusted screening and treatment strategies for current patients with PCa.

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## 1. Introduction

A shift toward a higher proportion of clinically localized, well-differentiated cancer at diagnosis in the United States and Northern Europe is unequivocally related to the widespread use of prostate-specific antigen (PSA) for prostate cancer (PCa) screening. Thus, tumors detected by PSA-based screening algorithms are often characterized by small volume and low grade (Gleason score  $\leq 6$ ). Stamey et al and other investigators therefore proposed that in the 21st century, a PSA elevation may solely reflect the amount of coexisting benign prostate hyperplasia (BPH) rather than mirroring PCa growth, thereby challenging the widespread use of PSA for diagnostic or prognostic purposes [1]. However, others reported evidence to show that PSA levels have prognostic value also for men with localized PCa [2].

However, due to widespread PSA testing, the lifetime risk of PCa diagnosis has increased to 16%, whereas lifetime risk of dying from the disease remains at 3–4% [3]. This raises concerns of putative over-diagnosis and resultant over-treatment. Up to 30% of cancers treated by radical prostatectomy (RP) in the United States fulfill current tentative definitions of insignificant cancer. Those cancers, given their minimal risk of influence on the quality or length or life, are unlikely to require definitive treatment with curative intent.

With recent progress in unraveling the human genome, as well as improvements in high-throughput technologies such as mass spectrometry and microarray analyses, many novel targets were identified [4]. However, despite discovery of an abundance of novel biomarkers for PCa in blood, only few candidates were later confirmed to contribute important clinical value [5]. Although original reports often show a statistically significant association between a candidate marker and a clinical state, subsequent studies on the same or related targets yield conflicting conclusions. Methodologic differences, poor study design, lack of standardized assay protocols, disregarding pre-analytical or analytical variation, or inappropriate and even misleading statistical analysis have been cited as explanations for these discrepancies [6,7]. Another issue relates to inadequate study populations, which in retrospective studies are often restricted (or biased) toward patients with available (“convenience”) blood samples. To allow an objective assessment of the quality of a study, reporting guidelines have been developed such as “REporting recommendations for tumor MARKer prognostic studies (REMARK)” [7] from the National Cancer Institute-European Organization for Research and

Treatment of Cancer (NCI-EORTC) working group on cancer diagnostics or the Surveillance Therapy Against Radical Treatment (START) statement for studies of diagnostic test accuracy [6]. These guidelines contribute relevant information about study design, preplanned hypothesis, standardization of assay protocols, and statistical analysis. The unanswered question in some studies of new markers is “Does the new marker significantly improve our ability to predict a clinical condition, given all the other currently available clinical parameters?” The answer to this question requires more than conventional univariable and multivariable analyses with associated *p* values. In our opinion, the performance of predictive models such as prognostic nomograms or artificial neural networks (ANNs) including or excluding any new putative biomarker, needs to show clinically significant improvement of accuracy to claim any real benefit [8–11].

Candidate biomarkers currently under investigation in bodily fluids other than blood, for example, PCA3 in urine [12] are not discussed in this article. Likewise we will not address whether the detection or characterization (using transcription profiling, fluorescence in situ hybridization, etc) of circulating tumor cells contributes important clinical value in men with PCa. This article focuses on current PSA-based strategies and discusses alternative candidate PCa biomarkers measured in serum.

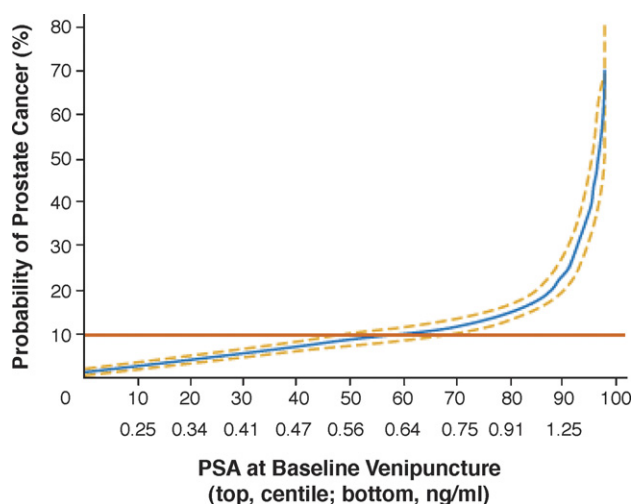
## 2. PSA-based strategies for PCa

Multiple large-sized population-based studies show unequivocal evidence that also only a modest elevation of the blood level of PSA above population-based averages is strongly associated with increased cancer risk [13–17]. However, it is also widely documented that the frequency of BPH increases sharply above age 50 and that it also causes the PSA level to rise in the blood. This helps to explain why PSA elevation may be common to both benign and malignant prostate disease, and why PSA is not a cancer, albeit tissue-specific, biomarker. Hence, a single PSA cut-off point may never contribute both high sensitivity and specificity although PSA is a powerful means to stratify population-based cancer risk. A cut-off point of  $\geq 1.0$  ng/ml (about the 80% centile for men aged  $\leq 50$  yr) may offer high cancer detection rates, but only modest to low specificity as opposed to a cut-off point  $\geq 10$  ng/ml, which offers very high specificity but very poor sensitivity in detecting PCa. More importantly, the cure rates are severely challenged for PCa cases with PSA levels  $> 10$  ng/ml at diagnosis.

Existing evidence that the commonly used PSA cut-off point of  $\geq 4.0$  ng/ml lacks both specificity and sensitivity illustrates important limitations of this arbitrary value, which lacks solid biologic rationale. In a prospective cohort study (Prostate Cancer Prevention Trial [PCPT]), designed to evaluate the preventive effect of finasteride, 15.2% of men (median age 69 yr) enrolled as untreated controls with PSA  $< 4.0$  ng/ml within 1 yr of the end-of-study biopsy harbored PCa, whereas high-grade disease increased from 12% at PSA of 0.5 ng/ml to 25% at PSA of 3.1–4.0 ng/ml [15]. Actually, median levels are approximately 0.7 ng/ml in the men aged 60 yr [16]. Hence, it is not unexpected that research has demonstrated a significant association between serum PSA levels of 4.0 ng/ml and PCa.

Elevation of PSA in plasma measured in men aged  $\leq 50$  yr (when coexisting BPH is less prevalent) was shown to be associated with diagnosis of PCa up to 25 yr later [16]. Compared to men with PSA  $\leq 0.50$  ng/ml, levels of 0.51–1.0 ng/ml at age 40–50 yr increased odds for incident PCa about 2.5-fold, corresponding to a long-term risk close to the population mean (about 10% by age 75 yr). For men with PSA in the range of 2–3 ng/ml, commonly defined as within the “normal” range, the odds for PCa increased about 10-fold (Fig. 1). Because PSA elevation at age 40–50 yr is more strongly associated with later diagnosis of PCa than an elevation of PSA at age 60 yr [17], a primary goal of PSA testing in men  $\leq 50$  yr should be to stratify cancer risk at an early age rather than PCa detection. This may enable a risk-adjusted screening strategy based on a single baseline PSA measured at age 40–50 yr.

Permutations of serum PSA measurements have been assessed in reference to their ability to improve



**Fig. 1 – Predicted probability of a prostate cancer diagnosis before age 75 years by population-based centiles of prostate-specific antigen (PSA) measured at age 44 to 50 years, with 95% CI. From Lilja et al [15].**

specificity and sensitivity of PCa detection. PSA velocity (PSA-V) has been proposed as another PSA permutation to achieve improved specificity. Initially PSA-V exceeding 0.75 ng/ml/yr was associated with higher risk of PCa than a slower rise in PSA over time [18]. Recent evidence suggests that this cut-off point is useful only for men with a total PSA  $> 4.0$  ng/ml. In younger men with a lower PSA level, PSA-V cut-off values of 0.3–0.5 ng/ml/yr were suggested as a basis for recommendation to perform a biopsy [19]. This study demonstrated that PSA-V may improve the predictive ability of a model incorporating PSA; however, results must be interpreted with caution due to verification bias in the study design. In a study from the PCPT, investigators demonstrated that PSA-V within 3 yr of diagnosis was predictive of PCa diagnosis [20]. However, when added to a predictive model that included PSA level, PSA-V did not add independent value in predicting cancer risk. Verification bias is not an issue in this study. Further weakness of serial PSA measurements is related to significant interassay variations and to significant physiologic between-day (biologic) variation of PSA levels, which has significant implications for screening and diagnosis [21–23].

D’Amico et al investigated whether pretreatment PSA-V could predict tumor stage, grade, and time to biochemical recurrence (BCR) after RP. This study reported significantly shorter time to PSA relapse and death from PCa in patients with an annual PSA-V of  $> 2.0$  ng/ml/yr among 1054 patients in the year prior to diagnosis [24]. The outcome prediction of an elevated preoperative PSA-V has been validated in other surgical series [25]. However, PSA-V has not been demonstrated to be an independent prognostic predictor of outcome after therapy when added to a model that includes PSA alone.

The discovery of various different molecular forms of PSA in the early 1990s facilitated the development of assays for selective immunodetection of PSA, which is not bound to plasma proteins [26,27]. By analyzing both free and total PSA (tPSA), the calculated percentage of free PSA (%fPSA) improves the potential for early PCa detection. Several single- and multi-institutional studies demonstrated enhanced specificity by using %fPSA for men with an elevated tPSA between 4 and 10 ng/ml and an initial negative prostate biopsy [28].

Complexed PSA (cPSA) determined by specific immunoassays has been suggested to improve specificity of PSA. For clinical use, a 3.2 ng/ml cut-off point for cPSA has been estimated to correspond to the 4 ng/ml tPSA threshold and seems to show similar diagnostic performance in early PCa detection [29]. However, most reports were unable to

show comparable diagnostic enhancements as those contributed by %fPSA, though some reported conflicting data could still warrant further investigation of cPSA [30].

A combination of immunochemical studies demonstrated that the structural composition of fPSA in serum differs measurably depending of whether it is released from hyperplastic or from cancerous tissue, that is, release of two principally different noncatalytic subfractions of fPSA: (1) incompletely processed single-chain forms retaining parts or most of the propeptide sequence (intact PSA, proPSA) [31,32] and (2) multichain forms that contain internal peptide-bond cleavages (nicked PSA, benign PSA [BPSA]) [33,34]. Some early reports on the putative clinical value of proPSA isoforms (eg, -7proPSA and truncated activation peptide proPSA forms, such as -2proPSA) have been suggested as enhancing specificity beyond that of tPSA and %fPSA [35] and to be associated with more aggressive cancer at PSA levels from 2–4 ng/ml [36]. By contrast, a European multi-institutional trial including 2055 men and immunoassays for some of the proPSA forms was unable to show any important increase in diagnostic accuracy over tPSA and %fPSA for early PCa detection [37]. Recent refinements and optimization of some of the assay protocols were reported by Väisänen et al, who demonstrated that technical flaws in the design of some of these assays may have confounded any evaluation of their putative clinical value [38]. However, it still remains to be shown whether such advancements are able to eliminate uncertainty of the clinical value of these measurements.

In summary, despite advances in the understanding of the molecular structure of PSA, its optimal clinical performance is unknown. Although a static trigger point of 4 ng/ml for early detection misses a significant number of cases, lowering the cut-off value increases PCa detection but, in parallel, it decreases diagnostic specificity by increasing the biopsy rate. Except for the use of %fPSA in early detection, none of the aforementioned PSA-based strategies have yet been sufficiently evaluated to warrant widespread clinical use. This clearly indicates the urgent need for novel biomarkers capable of improving specificity for early detection of non-indolent cancers.

### 3. Human kallikrein-related peptidase 2

Human kallikrein 2 (hK2 or KLK2) is another abundant “prostate-specific” serine protease that shares 80% amino acid sequence identity with PSA. It has predominantly a prostate-restricted expres-

sion pattern regulated by both the action of androgens and a functional androgen receptor (AR).

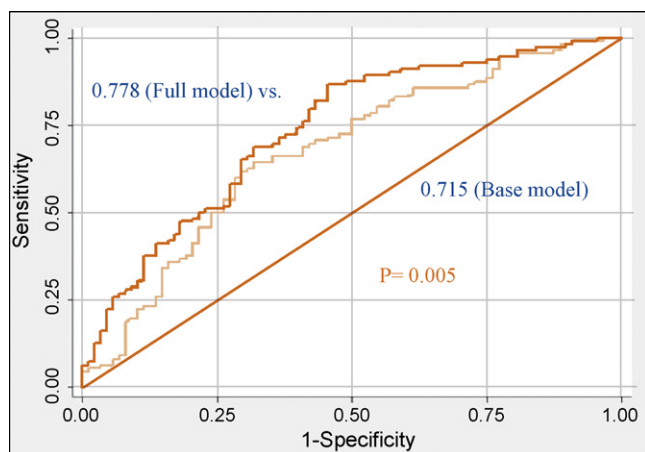
Extensive immunologic cross-reaction between hK2 and PSA and much lower abundance (about  $10^{-2}$ -fold) of hK2 than PSA may contribute to the reason very few data are reported from comparisons of tissue expression between hK2 and PSA. However, two studies have indicated that tissue expression of hK2 is higher in cancerous versus benign prostate tissue, whereas the cells expressing PSA are reported to be less frequent in cancerous versus benign tissue [39,40]. However, it is unclear whether this can be used to justify claims that hK2 is more closely linked to the biology of PCa than PSA. Nevertheless, recent reports using referral and screening cohorts suggest that improved discrimination of patients with or without cancer is accomplished by combining measurements of tPSA, fPSA, and hK2 compared to that of tPSA alone [41–43] and that such combinations enhance identification of insignificant tumors at RP [44]. Significant associations have been described between hK2 levels in blood, extracapsular extension (ECE), and PCa volume in RP specimens [45,46], and that hK2 provides independent prognostic information among men at risk for BCR after RP, particularly for men with tPSA  $\leq 10$  ng/ml [47]. In a cohort of 867 men, the predictive accuracy of hK2 for BCR was 72.1% versus 69.1% for PSA and 73.9% for hK2 versus 59.9% for PSA in men with a PSA  $\leq 10$  ng/ml. In addition, hK2 retained its independent prognostic ability for BCR when corrected for established clinical variables and also enhanced predictive accuracy when added to a standard prognostic nomogram [48].

In summary, the prognostic potential of hK2 becomes significant with features of progressing PCa, which was particularly marked in men with PSA levels  $\leq 10$  ng/ml. The clinical relevance of our findings is evident when one considers that in contemporary series a high proportion of men have a serum PSA  $\leq 10$  ng/ml. Particularly in the United States, there is a dramatic shift toward lower PSA levels at diagnosis due to widespread use of PSA testing for the detection of PCa. External validation studies are needed to confirm our findings on the prognostic role of hK2.

### 4. Urokinase-type plasminogen activator receptor forms

The plasminogen activation cascade has been reported to participate in degradation of the extracellular matrix during cancer progression. Urokinase-type plasminogen activator (uPA) binds to a specific receptor (uPAR) at the cell surface. Plasm-

nogen enhances conversion of uPAR-bound pro-uPA to active uPA. uPA is inactivated by formation of stable complexes with various plasminogen activator inhibitors (PAI-1, PAI-2, or PAI-3/PCI), whereas plasmin, uPA, and several other proteases cleave and release uPAR from the cell surface. As a result, full-length intact uPAR and cleaved isoforms are liberated from the cell surface by several mechanisms as previously described. These mechanisms provide a basis for the occurrence of detectable levels of soluble uPAR forms in plasma from healthy individuals. Elevated levels of various soluble uPAR forms in blood have been previously linked to prediction of prognosis in breast cancer, colon cancer, and non-small-cell lung carcinoma [49,50]. Recently, specific in-house research immunoassays were developed by Piironen et al and facilitated quantification of the individual forms of uPAR, comprising the intact uPAR, and domains I-III [51,52]. Recently, we studied the ability of fPSA isoforms, uPAR fragments, and hK2 to improve prediction of biopsy outcome among 355 patients with an elevated PSA [53]. As a result, uPAR fragments were significant univariate and multivariate predictors of PCa. Furthermore, PCa prediction was significantly enhanced by multivariate models supplemented by selective measurements of uPAR fragments and fPSA isoforms. Addition of these markers to a base model comprising tPSA and patient age improved the area under the curve (AUC) from 0.715 (base model) to 0.778 (model including uPAR fragments and fPSA isoforms;  $p = 0.005$ ; Fig. 2). Currently, a multi-institutional study is under way to confirm our initial findings.



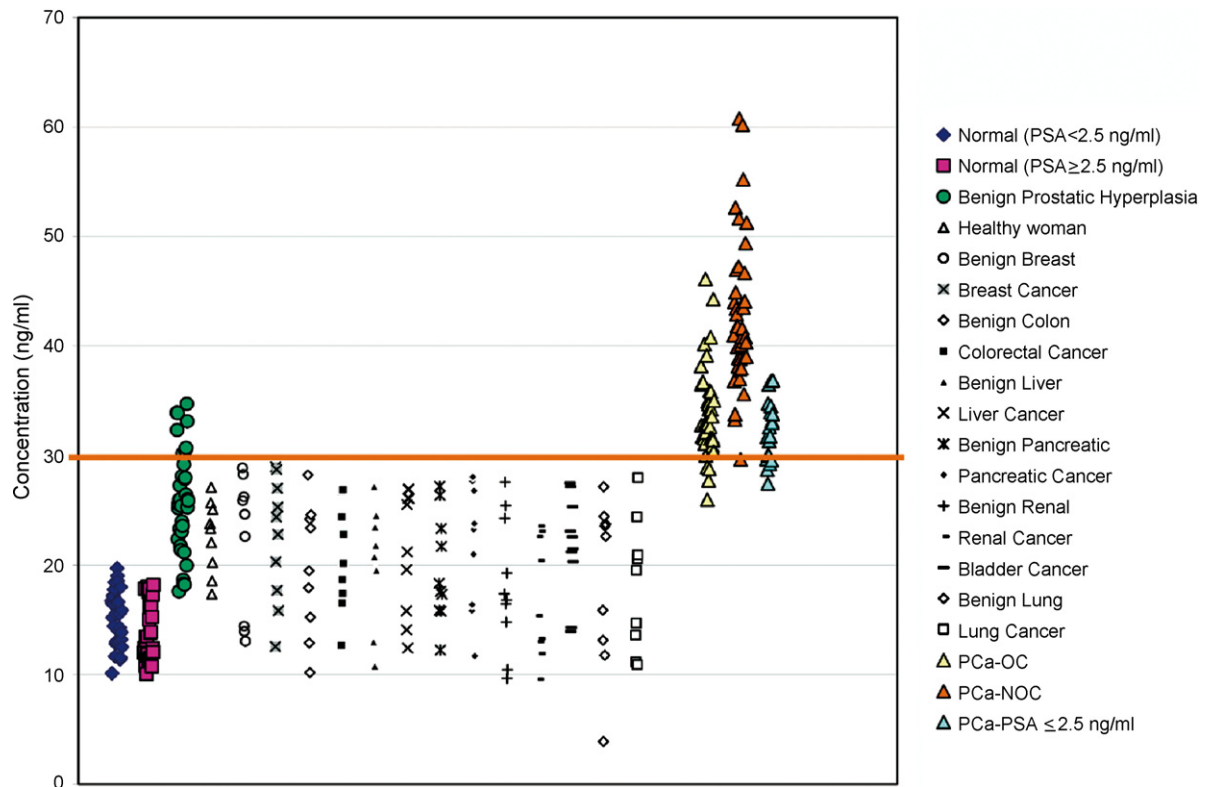
**Fig. 2 – Diagnostic accuracy (AUC) of a “base” model to predict biopsy outcome including age and pre-treatment total PSA and of the same model supplemented by free PSA subforms and various plasminogen activator receptor (uPAR) isoforms (full model) among 355 referral patients.**

Shariat et al also recently reported that plasma levels of uPA and uPAR are higher in men with PCa than in healthy controls and increase with disease progression [54]. Intriguingly, in their study, pretreatment serum uPA and uPAR levels were significantly associated with ECE, seminal vesicle invasion (SVI), and lymph node invasion (LNI) in multivariate analysis. Further, uPA independently predicted BCR in preoperative or postoperative multivariate models. Although these results are exciting, an essential next step would be to quantify that adding data on urokinase levels to currently available clinical data allows more accurate diagnosis or prognosis. Investigators would also need to show that these measurements improve decision-making.

## 5. Early prostate cancer antigen

The nuclear matrix is considered to be responsible for maintaining nuclear shape, function, and organization of its components. Alterations in nuclear matrix proteins have been demonstrated to be associated with carcinogenesis in multiple kinds of cancers. Two unrelated nuclear matrix proteins were implicated to be associated with PCa and, hence, called early prostate cancer antigen (EPCA) and EPCA-2. However, the structural composition of the corresponding target antigens detected by the EPCA or EPCA-2 antibodies still remains to be clarified.

Initial immunohistochemical studies using an antibody against EPCA revealed that biopsy specimens from men with PCa expressed a more intense EPCA staining compared to specimens from men with no evidence of cancer [55]. An enzyme-linked immunosorbent assay (ELISA) developed to measure blood levels of EPCA-2 using a novel anti-epitope antibody EPCA-2.22 was used to assess diagnostic cut-off points and the performance in discriminating PCa patients from healthy controls, though the authors did initially not report any technical or quality control data for the ELISA used to perform these measurements [56]. An EPCA-2 cut-off level of 30.0 ng/ml had a sensitivity of 94% for PCa diagnosis while maintaining 92% specificity (Fig. 3). In addition, EPCA-2 levels were differentially elevated in tumors with ECE as opposed to their organ-confined counterparts. Although the data certainly may be interesting, the study outcome should be used with caution because this clinical evaluation did not use representative population-based samples and the test performance needs to be assessed further. Therefore, larger independently generated validation studies are urgently required to confirm



**Fig. 3 – Serum analysis of EPCA-2 in a total of 330 serum samples screened for EPCA-2.22. Indirect ELISAs showed that EPCA-2.22 has cutoff of 30 ng/ml or greater at estimated concentration. Cutoff represented by the orange line across the graph. From Leman et al. [56].**

whether the early promising data using EPCA-2 serum measurements could be replicated by others.

## 6. Prostate cancer-specific autoantibodies

Similar to autoimmune diseases where autoantibodies are targeted against organ-specific antigens, several reports are available that describe the existence of autoantibodies against tumor-associated antigens, overexpressed in neoplastic cells. A humoral response to small amounts of tumor-related antibodies can be amplified through the production of high-affinity antibodies and T cells. These indicator proteins can be measured in trace amounts in the circulation. The existence of autoantibodies against PCa-specific antigens has been immunodetected in blood such as Huntington-interaction protein 1, prostasomes, and  $\alpha$ -methyl-acyl-coenzyme A-racemase (AMACR) [57,58].

Recently, Wang et al reported the use of a technique that combines phage display technology with protein microarrays to identify and characterize new autoantibody-binding peptides derived from PCa tissue [59]. This novel approach, termed

“cancer immunomics,” allows a global analysis of the humoral response against specific antigens in neoplasms. Samples from PCa patients and healthy controls were initially tested on a 2304-phage-peptide microarray, which allowed identification of 186 phage peptides with the highest level of differentiation between cancers and controls. These candidate peptides were then used to develop focused microarrays for analyses in the subsequent training and validation phase. As a final result, a 22-phage-peptide detector was designed from the training set, capable of discriminating 68 PCa serum samples from 60 healthy controls with 88.2% specificity and 81.6% sensitivity (AUC = 0.93), whereas PSA was 80% accurate (AUC = 0.80). Further studies are currently underway to validate this elaborate detection tool on a larger cohort and to further investigate the prognostic role of autoantibodies against cancer-related antigens.

## 7. Transforming growth factor- $\beta_1$ and interleukin 6

Transforming growth factor  $\beta_1$  (TGF- $\beta_1$ ) is a pleiotropic growth factor that regulates several cellular

mechanisms such as proliferation, angiogenesis, immune response, and cellular differentiation. Experimental PCa models have demonstrated the potential role of TGF- $\beta_1$  in the process of tumor cell progression. Subsequent studies have demonstrated an association between increased tissue levels of TGF- $\beta_1$  and grade, stage, and LNI in patients with PCa. TGF- $\beta_1$  can also be detected in the circulation by using a commercialized quantitative sandwich enzyme immunoassay, which does not cross-react with TGF- $\beta_2$  and TGF- $\beta_3$ . Several studies are available that reported a significant correlation of TGF- $\beta_1$  with ECE, SVI, and metastasis to bone and lymph nodes in men treated with RP for PCa [60]. Others, however, were not able to describe a significant correlation [61].

In vitro and in vivo studies have shown that human PCa expresses both interleukin 6 (IL-6) and its receptor (IL-6R), allowing for establishment of an autocrine/paracrine loop [62]. Elevated circulating levels of IL-6 and soluble IL-6R have been associated with features of aggressive PCa [63]. Based on these findings, Kattan et al developed and internally validated a prognostic model that adds plasma TGF- $\beta_1$  and soluble IL-6R to standard clinical predictors. Addition of novel markers to the nomogram improved the prediction of BCR by a prognostic substantial margin over results of the standard preoperative nomogram (predictive accuracy 75–84%) [9].

## 8. Proteomics

Proteomic serum profiling has been proposed as a novel diagnostic tool in PCa research. Changes in the composition of the proteins in serum may reflect the biology of various tissues and therefore may be used to differentiate patients with malignant conditions from those with benign disease. Promises arising from the initial profoundly discriminating sets of data [64] were later strongly challenged due to concerns for important biases regarding lack of standardization in the collection and processing of samples, analytical protocols, but also the interpretation after the analytical process [65–68]. A recent study demonstrated that a series of informative peptides present in serum processed according to a highly standardized protocol could efficiently discriminate between three different types of metastatic cancer in samples from patients with either prostate ( $n = 32$ ), bladder ( $n = 20$ ), or breast ( $n = 21$ ) cancer, and discriminate from the proteomic profiles found in healthy volunteer controls without cancer ( $n = 33$ ). This was then validated with an

external group of patients with PCa ( $n = 41$ ). Sixty-one signature peptides fell into several tight clusters thereby conferring cancer type-specific differences [69]. Further research may refine these signature proteins to allow identification of a surrogate marker for detection and classification of disease.

However, a recent multi-institutional consortium report was unable to discriminate men with PCa ( $n = 181$ ) from men with BPH ( $n = 143$ ) or healthy controls ( $n = 220$ ) using surface-enhanced laser desorption/ionization-based serum proteomic profiling [70]. A companion report provided evidence that the data, unlike previous studies, were devoid of pre-analytical biases [71].

## 9. Conclusions

The utility of serum-based circulating targets for PCa detection may result from disease-specific activation of various tissue protease cascades in the prostate gland, which presumably translates into a distinctive profile of biomarkers in blood detectable before the disease is clinically evident. Current evidence suggests that no single analyte is likely to achieve the desired level of diagnostic and prognostic accuracy for PCa. However, the combination of biomarkers with clinical and demographic data, for example, using established standard nomograms or ANNs represents a promising approach toward the goal of both optimal screening strategies and prediction of the natural course of a tumor. Furthermore, disease-specific molecular patterns can be derived from high-throughput technologies with remarkable affinity to malignant prostatic disease. Current studies demonstrate that understanding dynamic PSA changes over time may offer diagnostic and prognostic information, but PSA dynamics has not been demonstrated to be better than a simple static PSA cut-off value. In addition, PSA analysis in men <50 yr may represent a more intelligent strategy for early detection or risk stratification rather than focusing on older men in whom BPH becomes more prevalent. Bridging the gap between basic science and clinical practice represents the main goal in the near future to enable physicians to tailor risk-adjusted screening and treatment strategies for current patients with PCa.

## Conflicts of interest

Dr. Hans Lilja holds patents for free PSA and hK2 assays. Dr Lilja is also named as co-inventor on a patent application for intact/nicked PSA measure-

ments. Dr. Steuber and Dr. O'Brien have nothing to disclose.

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