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On the use of prostate-specific antigen for screening of prostate cancer in European Randomised Study for Screening of Prostate Cancer

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ABSTRACT

Prostate-specific antigen (PSA) has been the main drive for early detection of prostate cancer (PCa), including in population-based screening as in the European Randomised Study for Screening of Prostate Cancer (ERSPC). The specificity of PSA to indicate men with biopsy detectable prostate cancer can be improved by adding information obtained by new biomarkers, such as PSA isoforms. This improvement is needed to increase the efficacy of the screening procedure for the population-based as well as the individual screening. Various PSA isoforms, kallikreins and molecular markers have been validated in various cohorts from ERSPC of men with and without PCa in order to design the optimal diagnostic procedure for screening asymptomatic men. So far, most promising results have been obtained from the analysis of free PSA, proPSA, nicked PSA and hK2. The use of free PSA in addition to total PSA reduces the number of negative sextant biopsies at a PSA cut-off level of 3 ng/ml at initial screening with 30%, at the cost of losing 10% of detectable cancers that are predominantly well differentiated on histology. Further addition of PSA isoforms and hK2 only improve ROC curves in selected samples by a maximum of 5%. Molecular markers like PCA3 and TMPRSS2 in urine do not appear to be useful but they have been assessed insufficiently so far. The level of PSA at initial screening is highly predictive for the chance of being diagnosed with PCa later on in life. The changes in PSA over time after initial screening (like PSA-velocity and PSA-doubling time) are statistically different between men with detectable cancers versus those without (PSA-doubling time 5.1 versus 6.1 years), but this does not contribute significantly to population-based screening overall. Changes in specificity need to be related to a cost efficacy evaluation in the final analysis of ERSPC.

1. Introduction

Prostate-specific antigen (PSA) may be considered as the most remarkable tumour marker of the last decades. Remarkable because of the extra-ordinary scientific attention devoted to show its biochemical characteristics, epidemiological and clinical value and remarkable because of the huge commercial interest. In addition,

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also in the lay press PSA became a much debated item.[1,2]

This article describes first of all the use of total PSA levels in the blood from participants in the European Randomised Study of Prostate Cancer (ERSPC) as a criteria for prostate biopsy. Then, in addition, a number of studies with several candidate biomarkers conducted in the context of the PSA study group of the ERSPC on serum samples of ERSPC participants are evaluated.

From the beginning in 1994 total PSA cut-offs have been used in the ERSPC study to initiate for prostate biopsy. During a number of feasibility studies, the PSA cut-off was 4.0 ng/ml, based on a limited epidemiologic study,[3] describing 4.0 ng/ml as the upper 95% value in the normal population. Later on, this ERSPC biopsy cut-off was, for various reasons, changed to 3.0 ng/ml.[4,5] As of 2009 we were very aware of the biologic variation of PSA in the general population over time,[6,7] and that there was no PSA value that excludes the possibility of finding a prostate cancer by biopsy.[8] Nevertheless, during the complete period of the whole ERSPC study the PSA cut-off for biopsy played a predominant role with respect to the screening outcome, and therefore quality control of the PSA-test performance in the various European ERSPC centres was performed.[9]

Evaluation of the association between levels of total PSA in the blood and biopsy outcome among previously unscreened men with elevated PSA (e.g. $\geqslant$3 ng/ml) shows that PSA has moderate predictive value for biopsy detected PCa, as the positive predictive value is between 20% and 25% for a PSA value of 4 ng/ml, and increases to 50% 5 for a value of 10 ng/ml.[10] However, PSA retains little if any ability to discriminate men with from those without evidence of PCa at biopsy among men with elevated PSA who previously were subject to PSA-based PCa-screening,[10–12] and its PPV remains between 10% and 20% for all PSA values between 3 and 10 ng/ml. Although PSA measured before age 50 is strongly associated with subsequent diagnosis of PCa with unfavourable features[13] and PSA measured at age 60 is strongly associated with metastases or death from prostate cancer among unscreened men, the analysis of the cancers removed by radical prostatectomy and subsequent follow-up showed limited prognostic value of PSA. With the recent first outcome of the ERSPC on the beneficial effects of PCa screening on cancer specific mortality, there are urgent needs for additional biomarkers that may aid to reduce the large number of unnecessary prostate biopsy, which based on the annual incidence and annual number of biopsies can be estimated to be $\approx$750,000 in the US alone.[14,15] As approximately 50% of cancers detected by screening of the general population appears to be indolent,[16] there is also a need for biomarkers that may enhance predictions as to whether a biopsy-detect cancer may rather be left untreated, and as such pose little if any threat to the quality or length of life of the individual tumour host. Ideally, such tumours would be deferred from biopsy and, hence, could be left undiagnosed which is likely to improve the quality of life for this individual. Various candidate biomarkers, suggested to increase the performance of PSA as a diagnostic and prognostic marker, are still in transit in the long process of validation towards clinical application.[17] Understanding this process is illustrated by the history of the development of PSA, especially with respect to its role in ERSPC. The role of imaging techniques for diagnosis and staging is only mentioned briefly where relevant.

2. PSA development during ERSPC 1994–2009: total PSA

The development of PSA as a marker for the detection of prostate cancer has been fairly rapid. Early investigations on the antigenic properties of prostatic tissue started some 40–50 years ago.[18] The name prostate-specific antigen was first suggested by Wang et al.[19] During the 1980s of the last century broad clinical interest evoked, especially after the launch of the first commercial assays for PSA, Hybritech Tandem-R that was cleared by the FDA in 1985 to monitor prostate cancer after diagnosis. Subsequent studies reported by Stamey et al.[20] and Catalona et al.[3] contributed importantly to the fact that the PSA-test was also cleared by the FDA in for the detection of prostate cancer. During the period 1980–2000, important knowledge was gained on the biochemistry of PSA: PSA was found to be a kallikrein-like serine proteinase (*KLK3*) liquefying the ejaculate by degradation of the major gel-forming proteins (*SEMG1* and *SEMG2*) from the seminal vesicles.[21] Subsequently, the amino acid sequence of PSA was reported,[22] and catalytic PSA was shown to form stable complexes with several of the major extra-cellular antiproteases, e.g. alpha-1-antichymotrypsin (ACT; *SERPINA5*) and alpha-2-macroglobulin (*A2M*)[23] contributing severe restrictions to the binding of monoclonal antibodies to particularly one important epitope-region of PSA that provided the basis for the discovery of the testing for the free, unbound forms of PSA in the blood.[24] Notably, independent evidence showing that the major form of PSA in the blood was complexed to ACT was reported by Stenman et al.[25]

These discoveries led to two major subsequent developments:

1. the PSA standardisation project as initiated by Stamey et al. (Stanford, USA)[26] resulting in the acceptance of the so-called Stanford Calibrators for free and total PSA as WHO reference preparations in 1999[27] and
2. the report of an extensive PSA antibody epitope mapping[28] and the subsequent report based on a program as organised by the International Society for Developmental Biology and Medicine (ISOBM).[29]

The standardisation project became important because of the discordance between the many rapidly developed commercial PSA-assays.[30] It was partly due to variable recognition of complexed and free PSA and to the use of different calibrators and standards. The acceptance of the WHO standard with a 90:10 ratio, and the selection of antibody pairs that recognised free and complexed PSA equally solved this problem.[31]

In the ISOBM PSA antibody selection programme, 83 antibodies against PSA were studied revealing important information on the epitope specificity of many antibodies used in commercialised PSA-assays. This study formed the basis for the improvement of existing assays and the development of new ‘equimolar’ assays.

Table 1 – Beckman (access) versus Hybritech Tandem-E (Rotterdam, 2000).

Samples	Equation	Number	Agreement
Screening (1)	y = 1.04x	132	Yes
PCa (2)	y = 0.98x	288	Yes
NED (2)	y = 0.96x	87	Yes

Undefined screening (1) and well-defined clinical samples (2): prostate cancer (PCa) and no evidence of disease (NED). Range: 0–6 μg/l.
All comparisons: statistics according to Passing and Bablok[101] and Bland and Altman.[102]

Both these large international efforts resulted in considerable improvement of analytical performance of PSA measurements. The inter-assay variation for total PSA measurements is now 3–5% as compared to 10–15% in the beginning of the 1990-ties. Furthermore, the detection limit of 0.3–0.5 ng/ml around 1990 is now in the range of 0.01–0.02 ng/ml. Finally, the between method variation has shown a considerable improvement: from 25% to 30% in the early 1990-ties to 10–15% today according to the information of the Dutch Quality Assessment Foundation.

In the ERSPC this was reflected in the quality control programme for PSA started in 1998 based on the determination of total PSA – which approximately equals the sum of the free and complexed PSA forms – in two quality control samples every 2 months.[9] While the imprecision of the group initially was 10% with extreme values per participant of 3% and 24%, the mean score in 2006 was 5% and the range 2–8%. Part of the improvement can be ascribed to the introduction during 2000/2001 of the fully automated Access analyser in all ERSPC centres (Manufacturer: Beckman Coulter Inc.).

While in 1994 total PSA was measured by Hybritech Tandem-E, the enzyme immunoassay variant of the Tandem-R assay with the same antibody pair and the same calibration procedure and giving equivalent results,[32] in 2000, this method was replaced by the Access automated analyser also using the same antibody pair and the original Hybritech calibration procedure. Correlation between the two methods was excellent, as found in Rotterdam and confirmed elsewhere (Table 1).[33]

The changes in calibration policy did not affect the PSA measurements within the ERSPC study, as the Access PSA was calibrated against the original Hybritech standards. In 2006 Beckman Coulter changed their calibration policy for PSA by offering, in addition to the original Hybritech calibration, a calibration based on the WHO standards. A difference of 20% in test results was stated by the manufacturer and confirmed in a number of clinical studies.[34] A comparative study in 106 sera of unscreened and asymptomatic men selected from the Rotterdam database of the ERSPC showed a regression equation of PSA-WHO = PSA-Hyb * 0.796 + 0.007. Determination of cut-off values based on the WHO standards in the ERSPC would have resulted in a 30% decrease in the number of biopsies, with an identical decrease in cancers detected, while the characteristics of these cancers would hardly differ.[35] The warnings for clinicians to be aware of these issues have been published widely, as standardisation has an important effect on the interpretation of early detection studies. It is likely that many physicians are unaware that the calibration of the PSA-assays has changed over time. Within ERSPC it was decided to continue with the original Hybritech calibration in order to maintain the same biopsy cut-point as during the previous years.

During the timeframe of ERSPC 1994–2000 several developments took place which are noteworthy to mention here:

1. the discovery of complexed and free PSA forms in 1991 by Lilja and Stenman, and the availability of commercial assays regarding these forms several years later (see below) and
2. the description of assays for novel candidate markers: hK2 (kallikrein-related peptidase 2; *KLK2*), and free PSA subfractions (intact/nicked PSA and proPSA).

3. PSA forms, volume- and time dependent adjustments: diagnostic value for screening

The discovery of PSA forms became increasingly important for the early detection of PCa, as the initial ERSPC study results, based on total PSA only, indicated that a considerable number of biopsies resulted in a negative outcome, showing a low specificity of the PSA cut-off for a positive prostate biopsy, with a PPV of around 20% for a PSA cut-off of 3 ng/ml. Additional factors to improve the specificity were needed and possible by utilising the PSA forms.

As discussed earlier, reports published in the early 1990s showed that PSA does not only circulate as an inactivated enzyme in the blood as the result of leaking of PSA from prostatic epithelial cells, but also had the propensity to bind to various serum compounds, e.g. ACT and A2M, and the PSA-ACT complex constituted the major form of PSA in the blood.[23–25] It was discovered that the proportion of the PSA-alpha 1-antichymotrypsin complex was higher in patients with prostatic cancer than in those with benign hyperplasia and that the proportion of the free, unbound form of PSA was lower in men with prostate cancer than in those with benign hyperplasia.[36] Therefore, assay of the PSA-ACT complex had somewhat higher specificity for cancer than an assay for total PSA immunoreactivity,[25,37] findings which subsequently were independently replicated by other investigators.[38]

This finding became a source for a number of prospective and retrospective analyses on the specificity of markers. ERSPC maintained total PSA as the sole indicator for biopsies during the complete duration of its study. However, the size of the study allowed for a limited number of prospective side studies to analyse the value of alternative screening concepts with new markers (Table 2).

PSA changes over time were amongst the first to be analysed after sufficient follow-up.[39] In order to assess a shorter rescreen interval, a subset of 984 consecutively biopsied participants in the Rotterdam ERSPC cohort was analysed 1 year after first screening. This set up was inspired by the various protocols for repeat screening. While in the ERSPC a 4-year standard screen interval was chosen, in the Swedish ERSPC cohort a 2-year interval was maintained as of the earlier start of the protocol in 1995.[40] In the American PLCO study, a

Table 2 – prospective side studies within ERSPC on PSA related diagnostics.

Ref.	Parameter for Bx	PSA range	Result
Rietbergen et al.[39]	Rescreen 1 year	>4	No benefit of early rescreen
Roobol et al.[43]	PSA-doubling time < 4 years, PSA-velocities	1–2	No independent predictor for cancer
Recker et al.[44]	FT ratio < 20%	1–3	Low cancer specificity of FT ratio
Finne et al.[45]	FT ratio	<3	%fPSA < 15 strong predictor of later Pca
Raaijmakers et al.[46]	Total PSA	2–3	%fPSA predictive for aggressiveness, NOT for pos Bx

rescreen of 1 year is used,[41] while in the non-randomised screening cohort of the Catalona studies repeated screening takes place after 3 months.[42] In the Dutch side study with a screening interval of 1 year, and an absolute PSA biopsy indication of 4.0 ng/ml no benefit was observed by shortening the rescreen interval.[39]

The PSA-doubling time as an indicator for repeated (first rescreen round) biopsies was assessed in a prospective side study of 113 men in the second round of the Dutch study site in the low PSA range between 1 and 2 ng/ml.[43] Doubling of the PSA concentration within the 4 years, or any other increase of PSA (PSA-velocity) did not contribute to the prediction of a detectable cancer.[10] Although statistically a difference was found between men with versus without cancer in mean PSA-velocity (0.62 ng/ml/year versus 0.46 ng/ml/year, $P = 0.001$), and in mean PSA-doubling time (5.1 years versus 6.1 years, $P = 0.002$), the variability of these parameters for individual decisions would be too high for practical application. In a multivariate analysis of a comparable cohort the odds ratio for the PSA-velocity was 0.73 (95% confidence interval (CI), 0.20–2.6, $P = 0.64$).[43]

The ratio of free-to-total PSA (F/T ratio or %PSA) was a second factor analysed prospectively in two cohorts from Aarau and Helsinki, in a PSA range below 3.0 ng/ml in order to assess the sensitivity and specificity of various F/T ratios as an indicator for biopsy. The smaller Swiss study[44] showed that amongst 158 (94%) patients who underwent a prostate biopsy, a prostate cancer was detected in 17 (10.8%). There was no increase in specificity of any parameter in that PSA range. The Finnish study[45] demonstrated in 17,680 participants with a follow-up of 5.8 years that a low %fPSA (less than 15%) was a strong predictor of later diagnosis of prostate cancer. Men with a %fPSA in the lowest quartile (<14.2%) showed a 6.9-fold risk compared with those with a level in the highest quartile (>23.7%).

Lowering the indication for biopsy to a PSA level of 2 ng/ml provided information on the true incidence of biopsy detectable prostate cancer in the general population.[46] Simultaneous analysis of the FT ratio in this cohort did not show any diagnostic benefit as a biopsy indication, but provided data on the potential low prognostic value of the FT ratio in this total PSA range.

In addition to these prospective studies, retrospective studies on the various PSA forms (%fPSA, intact/nicked PSA, proPSA, etc.), dynamic changes in PSA concentrations over time (PSA-velocity or doubling time), and hK2 have been analysed retrospectively for their ability of adding information to that provided by total PSA. No urine samples were collected in the past that could allow any of the ERSPC-investigators to perform a retrospective analysis of the value of the PCA3-test. However, during the third and fourth round of ERSPC in Rotterdam urinary samples were collected and analysed in a prospective manner to enable evaluation of the ability of the PCA3-test to predict biopsy outcome amongst men who had recently been subjected to PSA-screening.

Such studies inherently suffer from attribution bias, as no combination of markers is tested prospectively, and therefore especially false-positive/negative results in sera with a total PSA less than 3 ng/ml (the biopsy cut-off) cannot be evaluated properly in the absence of biopsies, the so-called verification bias. Combinations of marker data with clinical information, i.e. prostate volume and digital rectal examination (DRE) were evaluated by conventional logistic regression analysis and neural networks. In Finland, the expertise to build a neural network was used to show the benefit to predict positive biopsies in men with a total PSA above 4 ng/ml.[47] The Finnish design of a neural network for screening in the general populations was validated in a Swedish and Dutch cohort,[48] in which a prospective setting was simulated by dividing the data set chronologically into one set for training and validation (67%, $n = 1183$) and one test set (33%, $n = 592$). The diagnostic models were calibrated using the training set to obtain 95% sensitivity. When applied to the test set, the Logistic Regression (LR) model, the Bayesian regularisation (BR-MLP) model and the proportion of free PSA reached 92%, 87% and 94% sensitivity and reduced 29%, 36% and 22% of the false-positive PSA results, respectively. At a fixed sensitivity of 95% in the test set, the LR model eliminated more false-positive PSA results (22%) than the proportion of free PSA alone (17%) ($P < 0.001$), whereas the BR-MLP model did not (19%) ($P = 0.178$).

Based on the retrospective analysis, implementation of %fPSA in a population-based screening would have resulted in a considerable increase in specificity at the cost of a small loss of sensitivity. In a large study of 1726 participants, the number of false-positive indications could have been reduced by 37% by the use of a %fPSA cut-off of 16% or less as a biopsy indication, at the cost of missing 11% detectable cancers.[49] When restricting the use of %fPSA to the PSA range of 3–10 ng/ml, the reduction became statistically significant in the overall ROC curves.[50] In the Swedish population of 9973 participants the cancer detection would have been 14% higher with a PPV of 36% using a threshold tPSA of > or =3.0 ng/mL combined with a %fPSA threshold of < or =18%.[51] %fPSA therefore enhances prediction of biopsy outcome in previously unscreened but also previously screened men.[12,52]

These studies showed a similar pattern for other markers and parameters like digital rectal examination (DRE),

prostatic volume and age, but that these were less powerful or even insignificant compared to the FT as supplements to PSA in order to increase specificity. Prostate volume was evaluated by calculating PSA-density, i.e. the correction of total PSA for prostate volume measured by ultrasound (US) and even PSAT, which is the correction of PSA for the ultrasound measured transition zone volume.[53] The variability of the ultrasonic measurements explains partly the low impact on diagnostic procedures.[54]

It is important to note that during 1990s the number of cancers detected by sextant biopsy were analysed for their diagnostic value at initial screening, and the relative improvement of sensitivity or specificity, and that tests (or combinations of tests) were compared using ROC curve for analysis for various ranges of PSA. This analysis did not reflect the statistical or clinical relevance at predetermined cut-offs of sensitivity, nor did they include tumour characteristics or clinical outcome over time.

PSA-velocity over a limited number of observations and follow-up rounds was assessed in various studies. In 588 consecutive participants in the Dutch ERSPC cohort that presented at their first screening with PSA values <4.0 (none biopsied) and who progressed to PSA values >4.0 ng/ml 4 years later (all biopsied), PSAV did not improve the detection characteristics of a PSA cut-off of 4.0.[55] The lack of predictive value of PSA-velocity for a positive biopsy was confirmed in a later study of over 2200 men after two screening rounds.[56] Recent reports based on the ERSPC-participants in Rotterdam and in Sweden found that although certain PSA-velocity or doubling time definitions independently associate with biopsy outcome, there is little (if any) evidence that PSA-dynamics contribute important discriminatory value beyond that of single total PSA value alone.[57]

For DRE, a follow-up of 8 year after initial cancer-negative biopsy, an initially suspicious DRE did not influence the chance for detection of cancer or significant cancer at later screens.[58] This confirms the initial reports on the serendipity and poor diagnostic value of DRE.[59] Changes in %fPSA over time also have been assessed, and did not show of importance predicting the outcome of biopsies when compared to that of a single value of %fPSA alone.[57] However, interestingly the long-term predictive value of PSA and isoforms was assessed retrospectively in a large, representative cohort of Swedish men with >21,000 participants in a cardio-vascular study providing anti-coagulated blood plasma at age <50 that was stored frozen for over two decades.[60] A tPSA increase of 1 ng/ml was associated with an increase in odds of cancer of 3.69 (95% CI, 2.99–4.56); addition of DRE information on palpable disease, or other PSA forms or hK2 did not add to the predictive value of tPSA.

4. hK2 (Kallikrein-related peptidase 2), and free PSA subfractions (intact/nicked PSA and proPSA)

As the ERSPC team is closely related to the clinical and scientific laboratories for the development of PSA related markers of Profs. Kim Pettersson, Timo Lövgren, Hans Lilja and Ulf-Hakan Stenman, a continuous evaluation of improved assays took place. Next to the determination of PSA (also known as kallikrein-related peptidase 3) in its free and complexed forms, new kallikrein family members closely related to PSA (e.g. kallikrein-related peptidase 2 or hK2),[61] and differently processed forms of the non-catalytic PSA-molecule were subject to characterisation in further detail. hK2 was not only found to be genetically and structurally highly similar to PSA (i.e. 80% amino acid identity), but it also appeared primarily localised to the epithelial cells of the prostate.[62]

Reports suggested that hK2 could be more strongly associated with prostate tumours than PSA,[63] and highly expressed in poorly differentiated cancer cells.[64] It was found that hK2 converts the inactive precursor form of PSA, proPSA rapidly to active PSA.[65,66] The high homology between hK2 and PSA resulted in significant cross-reactivity of hK2 with polyclonal and some monoclonal antibodies to PSA,[67] although the discovery that certain epitopes were unique for each of these molecules enabled the first design of an immunoassay specific for hK2.[68] Several subsequent improvements of the assay-protocol for hK2 have been reported,[51] compared and standardised against the critical reagents and assay-protocols developed by other groups of investigators.[69] Based on data from the ERSPC study in Sweden, hK2 can be used in a panel of markers to reduce unnecessary biopsies.[52] There is, however, also a prognostic value in hK2, as it is correlated to a number of intermediate (surrogate) endpoints: in 91 men with cancer that underwent radical prostatectomy from the Dutch ERSPC cohort, hK2 added prognostic value for the detection of minimal prostate cancer with Gleason 6 in those screen-detected cases within PSA range 4–10 ng/ml.[70] Based on differences in serum concentrations, hK2 seems to be a powerful predictor of organ-confined disease and pathologic stage of clinically localised prostate cancer, especially in the PSA range below 10 ng/ml,[71] or as a tool to improve discrimination between poorly differentiated and non-organ-confined prostate cancer.[72]

Free PSA (fPSA) in serum consists of a mixture of PSA variants,[6,73] comprising three distinct groups of inactive PSA (Fig. 1). One group, defined as proPSA, is a mixture of precursor forms of PSA. A second group comprises single-chain, non-catalytic forms that – similar to the above mentioned proPSA forms – manifests high-affinity binding to monoclonal antibodies detecting intact PSA.[74] The third group comprises non-catalytic forms that are internally cleaved[23,75]; either at an internal cleavage between Lys_{182} and Ser_{183} found at elevated concentrations in BPH patients and therefore called BPSA,[76] or nicked PSA that is cleaved C-terminal of either Lys_{145} and/or Lys_{146}.[74] Development of highly sensitive, specific assays that enable reliable assessment of these molecular forms requires novel monoclonal antibodies manifesting highly selective recognition of these heterogeneous subfractions of free PSA.[77,78]

Although proPSA was originally defined as a precursor protein of PSA consisting of 244 amino acids,[22] and therefore often called (–7)pPSA (compared to the mature catalytic form of PSA that has 237 amino acids), subsequent work showed that recombinant production resulted in release of both (–7), (–5) and (–3) forms of proPSA that manifested no catalytic action.[65] ProPSA is found in normal prostatic epithelium together with truncated forms. Mikolajczyk et al. identified

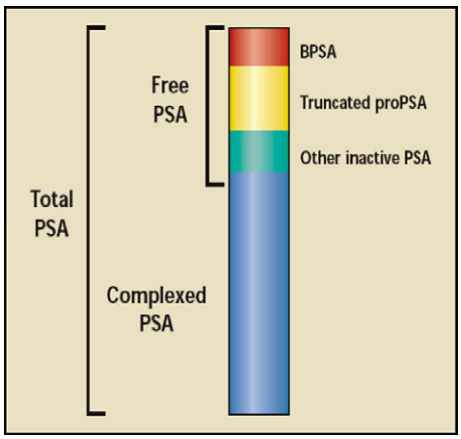

Fig. 1 – Schematic overview of PSA and isoforms.

a clinically important form, (–2)pPSA containing 239 amino acids. This form occurs in prostate cancer tissue and also in serum.[79] The various precursor forms of PSA have been suggested to contribute unique predictive values. The concentrations of (–7, –5)pPSA, hK2 and fPSA have been used to distinguish between BPH and cancer, but proPSA and hK2, alone or combined, did not improve the specificity of % fPSA for discriminating BPH from cancer.[48] [–2]pPSA, sum [–7, –5, –4, and –2]pPSA, and recently called just 'proPSA' (pPSA), had diagnostic value above %fPSA to distinguish BPH from PCa,[80] but limited prognostic value for cancers detected.[81]

The BPSA form (benign prostatic hyperplasia-associated PSA) is clipped between Lys_{182} and Ser_{183},[76] represents 25% of the fPSA in biopsy-negative men in serum, while its proportion is significantly higher in BPH compared to PCa serum.[82] Furthermore, BPSA outperformed %fPSA as well as tPSA in the prediction of transition zone enlargement and might therefore aid in discriminating PCa from benign prostatic hyperplasia (BPH).[83] By contrast to the other free PSA subfractions, there are, however, no data reported to indicate that BPSA can be used in a screening setting for this purpose.

Intact PSA, was discovered based on a previously unrecognised 3D-conformation-dependent epitope present in both proPSA and mature PSA, but which is lost when PSA is internally cleaved at Lys_{145} and/or Lys_{146}.[78] Calculating the difference between the serum or plasma concentration of free and intact PSA gives the nicked PSA (N-PSA) concentration, which has been proposed to possibly correlate with the TRUS volume of the prostate gland.[80]

The increasing amount of information on these PSA related markers together with the clinical parameters called for further assessment and integration into diagnostic and prognostic instruments that could serve the daily practice of early detection and screening for PCa.

5. Integrating information for different steps in the diagnostic process

Increasingly, the information provided by the evaluation of novel markers and parameters was integrated in order to validate their relative values during the various steps of the diagnostic process. With respect to PSA isoforms, the combination of the truncated forms (like pPSA) showed additional value over total PSA (tPSA) and %fPSA for PCa detection in the tPSA range of 2–10 ng/ml. At a high sensitivity for PCa, the specificity of the ratio of pPSA to fPSA (%pPSA) appeared, in general, better than that of the ratio of %fPSA with a gain of 5–11% in diagnostic accuracy.[84] In reference to other members of the kallikrein-related peptidase family, there are several studies suggesting that hK2 may have an additional diagnostic value in predicting PCa-risk compared to %fPSA alone. However, assays measuring any other kallikrein-related peptidases cannot be recommended.[85] Further, the degree as to which hK2 and intact PSA compared to pPSA may contribute diagnostic enhancements has not been adequately assessed.

Interestingly, it was reported that the information provided by some of the clinical parameters (like age or prostate volume) could be eliminated by the predictive information obtained from a serum parameter. Especially for parameters with a large intra- or inter-observer variation, like ultrasonic volume of the prostate, it appears to be an attractive option to replace them by serum markers, which in itself have a low coefficient of variation (CV).

For the nicked PSA fraction of free PSA this was reported in a set of serum samples that had been collected from men with negative biopsy ($n = 164$) and PCa ($n = 252$). Except the intact PSA fraction, all PSA fractions and hK2 were associated with transition zone volume of benign prostate hyperplasia (BPH) in multivariable linear regression analysis, while hK2 and the intact PSA fraction were the individual forms most closely associating with the volume of prostate cancer assessed after prostatectomy.[80]

Logistic regression models have been developed as an aid in predicting prostate biopsy outcome using recent, highly optimised in-house research versions of hK2- and intact PSA-assays[78] and the dual-label assay design[86] manufactured by Perkin–Elmer [Turku, Finland] to measure the free and total PSA levels in the blood. Using blood samples from 740 previously unscreened Swedish ERSPC-participants who underwent prostate biopsy due to elevated PSA ($\geqslant 3$ ng/ml),[52] the panel of four-kallikrein markers improved the diagnostic performance assessed as area under the curve (AUC) from 0.68 to 0.83 (compared to a base-model with only total PSA and age). Also, this panel also contributed enhanced accuracy in predicting high-grade prostate cancer (Gleason score $\geqslant 7$ at biopsy) from 0.82 (base-model) to 0.87 (kallikrein-panel), while the enhancements were moderate when also DRE was included in this prediction (from 0.87 to 0.90). Using the panel of four-kallikrein markers at a biopsy-threshold of 20% risk of prostate cancer would have reduced the number of biopsies by 424 (57%) and missed only 31 out of 152 low-grade and 3 out of 40 high-grade cancers.[52] A follow-up study of recently screened ERSPC-participants who underwent prostate biopsy due to elevated PSA during the subsequent rounds of screening illustrated clearly that the predictive value of total PSA is drastically impaired and is not much better than a 'coin-flip' in this setting.[52] However, the panel of four-kallikrein markers were similarly contributory in aiding prediction of biopsy outcome with the AUC increasing from 0.56 to 0.67, and the AUC increasing from 0.66 to 0.82 for evidence of high-grade cancer at biopsy. Importantly, these results have now also

been validated in the independent, representative, population-based cohort from the Rotterdam section of the European Randomised Study of Screening for Prostate Cancer (ERSPC).[87] The multi-kallikreins panel was found to predict PCa in men in the first round of a population screening study, reducing the number of biopsies by 413 per 1000 men with elevated PSA and missing only 60/216 low-grade and 1/43 high-grade cancer.

All of this supports the idea that multiple kallikrein forms and PSA isoforms measured in blood can predict the result of biopsy in previously unscreened men with elevated PSA. A multivariable model could determine which men should be advised to undergo biopsy and which might be advised to continue screening, and defer biopsy until there was stronger evidence of malignancy. On top of this, it was realised that the diagnostic parameters differed in their performance during the various steps of the diagnostic process. Previously, it was already shown that the predictive power of total PSA for a positive biopsy in the initial screening round dramatically decreased during the second and following screening rounds,[10] and that prior participation in PSA-screening significantly changes the performance of statistical models developed to predict cancer in unscreened men. These observations developed into a set of nomograms for population-based cancer detection that differentiates between the various diagnostic settings during screening and follow-up. This stepwise nomogram has been published as the ERSPC prostate risk calculator (http://www.prostaatwijzer.nl), that supports patients and clinicians in making decisions.[88] Important for them is to have a realistic estimate on risks of having a significant cancer. Apart from that, it is of course important to get an impression on the biologic behaviour of a tumour, once diagnosed.

6. PSA and prognosis

With the increasing number of detected cancers and their follow-up within the ERSPC study, more information on cancer characteristics became available. It was realised that nearly 50% of cancers detected showed the features of indolency (the identical pathological features found coincidentally at death due to other cause). These cancers represent 'overdetection', as there is no benefit of diagnosing these asymptomatic cancers.[16] The follow-up data were assessed in a retrospective setting, and therefore initially based on surrogate endpoints (as the mortality endpoint was not obtained). As Gleason scores are still regarded as the best surrogate for the biologic course of a prostate cancer, serum parameters were related to the Gleason score in prostate biopsies or the histology of cancer removed by radical prostatectomy. PSA and its derivative free PSA (fPSA) showed little correlation with histologic differentiation,[50] and therefore different assays did not selectively lead to the detection of subsets of prostate cancers.[89] There was only minimal support for a more differentiated recognition of poorly versus well differentiated cancers by the kallikreins[70] or by the truncated PSA forms.[84] Indolency remained therefore defined especially by the results of the diagnostic biopsies, like tumour length, number of positive cores and Gleason score.[90] The call for novel prognostic markers became louder with the observation of the increasing number of indolent cancer diagnosed.[91]

7. Marker discovery for screening of Pca

International collaborations for the development of prognostic markers had been initiated already in the eighties (e.g. BIOMED), but the scientific strategic agenda of the European Union facilitated the construction of large scientific consortia at the turn of the century. Under the umbrella of the Framework Six Program, the consortium P-Mark was launched for the discovery and validation of novel and close to clinical markers.[92] ERSPC contributed to this P-Mark programme that concentrated around the new proteomic technology and the initiation of an international retrospective and prospective biorepository. One of the preclinical markers that were to be evaluated was, next to the serum markers like intact PSA and hK2 mentioned above, the genomic marker PCA3 in urine.[29] PCA3 in urine is currently being validated simultaneously to the kallikrein panel in serum in a large cohort from the third screening round of the Rotterdam section of the ERSPC. Other proteomic markers are still in the early phase of development, are awaiting validation in selected serum samples.[93] It is often impossible to determine the fate of a candidate marker upfront, or where to position them clinically. Markers of neuroendocrine differentiation are useful for the monitoring of androgen-independent disease and various bone markers are useful in patients with metastatic disease, but they have little impact on the early diagnostic phase.[50,94,95]

The subject touches the hearth of current basic research activities in the Pca field, as validation of novel genomic or proteomic markers in tissue, blood, or urine is one of the major restrictions for development. There are many examples of candidate markers published, also with validation in subsets of ERSPC cohorts (e.g. in serum: insulin-like growth factor 1[96,97]); circulating cells in blood,[98] but most efforts stop at this level of validation without mutual comparison. The relative contribution from each candidate marker can only be established by a simultaneous assessment in a large population-based cohort with adequate clinical follow-up of 5–10 years. The development of repositories that facilitate cross validation is scientifically essential for an efficient use of research activity and resources. Limitation is the lack of adequate urine repositories and the still labour intensive isolation of genomic material from cancer cells in prostatic biopsies.

8. How to proceed? Conclusions

As the ERSPC data mature, more is known on the fate of men with and without cancers that started to participate in the study from 1992 onwards and are followed for the rest of their life. The predictive value of PSA as a mainstay and the advantageous effect of screening have recently again been emphasised by the study of Van Leeuwen, comparing a non-screened population with known PSA with a matched screened population in the ERSPC.[99] The PSA dependent risk on detectable Pca, and similar efficacy of screening are illustrated.

Due to the close follow-up the ERSPC study can show the predictive value of clinical and laboratory markers over time. Biorepositories allow for simultaneous retrospective assessment of markers in relation to final disease outcome. Especially patients in whom no or little/late intervention has taken place, for example, in men on watchful waiting or active surveillance, or those with BPH, it is useful to assess these serial serum markers. This is illustrated by the validation of the prostate risk calculators and the assessment of the long-term risk at an early age on a positive diagnosis of significant prostate cancer later in life, using total PSA, free PSA, %fPSA and hK2 in the MPP-cohort from Malmö, Sweden.[60,100] The development of high throughput genomic markers in biopsy materials is likely to contribute to the prognostic value of serum markers. However, the clinical assessment of serial changes over time in serum markers is a powerful tool for life long risk assessment. Algorithms based on the combined use of serum markers, clinical parameters and genomic assessments, whether by logistic regression or by neural networks will improve the diagnostic accuracy for prostate cancer and assays for minor subfractions of PSA and other new markers may provide additional prognostic information. The first candidates in priority are proPSA, intact PSA in serum and PCA3 together with TMPRSS2-ERG in urine, and their inclusion in a risk calculator that enables simultaneous assessment for the various diagnostic settings is needed.

PSA has made the ERSPC story. Or is it fair to say the opposite: ERSPC has improved our knowledge about PSA? The current data underline the optimal policy for screening PCa that a PSA value as an indicator for biopsy has to be interpreted with respect to prostate volume in the first place and less so by DRE information and age. PSA might well be in transit to a kallikrein panel but remains the most important component so far. It is likely that from such information, strategies for individual screening policies after first screening based on PSA will be developed.

Conflict of interest statement

Prof. U.H. Stenman is a co-author on a patent on free PSA, Prof. H. Lilja holds patents for free PSA and hK2 assays and is named as co-inventor on a patent application for intact PSA-assays. Dr. Blijenberg, Dr. Van Schaik, Dr. Roobol and Prof. Bangma declare not to have a conflict of interest.

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