Molecular Forms of Prostate-specific Antigen and Human Kallikrein 2 as Promising Tools for Early Diagnosis of Prostate Cancer

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Abstract

Prostate-specific antigen (PSA) is the most useful marker in the early detection of prostate cancer and for the monitoring of patients with this diagnosis. Molecular forms of PSA and also human kallikrein 2 have been used to discriminate between benign prostatic hyperplasia and prostate cancer as well as for the detection of prostate cancer within the gray zone of PSA. In this respect, a literature survey on the diagnostic validity of free PSA (fPSA) related to total PSA (tPSA), PSA bound to α1-antichymotrypsin (ACT-PSA), and complexed PSA is given together with our results. The ratio of fPSA:tPSA has been shown to improve the specificity of prostate cancer diagnosis on the basis of tPSA measurements. Unnecessary biopsies can be reduced by about 19–64% in the total PSA range of 4–10 μg/liter while only missing 5–10% of cancers. Furthermore, carcinomas in patients with PSA values <4 μg/liter can be detected, indicating an improved sensitivity because of the percent fPSA at low PSA values. ACT-PSA or complexed PSA alone and the calculated derivatives are not superior in their discriminatory power compared with the percent fPSA. The diagnostic significance of the other molecular PSA forms and human kallikrein 2 needs to be evaluated in more extensive clinical trials.

Introduction

PCa2 is currently the most common cancer and the second most frequent cause of cancer deaths in men in the United States (1). Approximately 180,400 new cases and 31,900 deaths from this disease are expected for the year 2000 (1). There have been many efforts to detect this malignancy in an early, curable stage and thus to reduce the mortality rate.

Since its discovery more than 20 years ago, PSA has been established as the most valuable tool for early detection, staging, and monitoring of PCa (2). In the early 1990s, at least five large series of studies clearly showed that for the detection of PCa, PSA determination alone is better than DRE or other parameters, and that the combination of PSA and DRE is the most effective way to detect PCa (3–7).

A large multicenter PCa screening trial of 6630 men showed that the positive predictive value of PSA increased from ~10% in men with PSA concentrations <4 μg/liter to >80% when the concentrations were >20 μg/liter (5). Most patients with a PSA value <10 μg/liter were diagnosed with early stage disease, whereas ~50% of patients with PSA concentrations >10 μg/liter had advanced disease. These data demonstrated the need for a low PSA cut-point for detecting PCa in early, curable stages. The generally accepted PSA cutoff of 4 μg/liter leads to a rather high number of 65% false-positive findings, demonstrating the inability of PSA to discriminate PCa from other benign diseases (8). This is because PSA is mostly organ- but not cancer-specific. Elevated PSA concentrations are also observed in BPH, PIN, prostatic ischemia or infarction, acute and chronic prostatitis, and after clinical manipulations (reviewed in Ref. 9). According to the PSA in serum, ~20% of all PCa patients have a serum PSA concentration <4 μg/liter at the time of diagnosis, representing the false-negative rate (3, 10). In addition, PSA also exists in various nonprostatic sources such as milk or nipple aspirate fluid from the female breast, or in perirectal, anal, and apocrine sweat glands (11–13).

Various methods were proposed for improving the sensitivity and specificity, especially in the range of 4–10 μg/liter PSA, to detect PCa. Several concepts such as PSA density, PSA transition zone density, PSA velocity, and age- or race-specific reference ranges have been developed to reduce the false-negative and false-positive rates (reviewed in Refs. 14–17). These calculations based on total PSA could not always fulfill the expectations to reduce the number of unnecessary prostate biopsies (15, 18).

Moreover, it has been shown that PSA in serum exists in different molecular forms, and that the measurement of these forms offers new possibilities to improve the diagnostic discrimination between PCa and BPH. The objective of this paper is to review all molecular forms of PSA in serum and tissue and the current views of their clinical utility. Additionally, another member of the serine protease family, the human glandular...
kallikrein 2, will be discussed separately as a possible new marker for early stage PCa.

### Molecular Forms of PSA

PSA circulates in serum in complexed forms (bound to protease inhibitors) or in uncomplexed (free or unbound) forms (19–21). tPSA represents 10–30% of tPSA. Approximately 70–90% of the tPSA is bound to the serine protease inhibitor ACT. Trace amounts of PSA are also bound to API (old nomenclature: α1-antitrypsin), ITI, pregnancy zone protein (an A2M-analogue), or PCI, whereby the PSA-PCI complex could only be detected in seminal plasma and not in serum (21–25).

Another fraction of the PSA is bound to the 25-fold-larger A2M molecule. Because of its large size, the A2M completely encapsulates the smaller PSA molecule, and no free epitope sites of PSA remain for its detection (26). The PSA-A2M can only be detected after the complex has been opened (27). A2M molecule. Because of its large size, the A2M completely encapsulates the smaller PSA molecule, and no free epitope sites of PSA remain for its detection (26). The PSA-A2M complex could only be detected in seminal plasma and not in serum (21–25).

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Table 1 gives a synopsis of all molecular forms of PSA described to date in serum, seminal plasma, and prostatic tissue.

<table>
<thead>
<tr>
<th>Molecular forms</th>
<th>Prostatic tissue</th>
<th>Seminal plasma</th>
<th>Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Carcinoma</td>
<td>Normal/BPH</td>
<td>PCA patients</td>
</tr>
<tr>
<td>tPSA</td>
<td>7.25 µg/mg protein (41)</td>
<td>20.2 µg/mg protein (41)</td>
<td>0.2–5 µg/liter, 30–50% enzymatically active (157)</td>
</tr>
<tr>
<td>tPSA</td>
<td>5.58 µg/mg protein (41)</td>
<td>15.1 µg/mg protein (41)</td>
<td>&gt;95% of tPSA (157)</td>
</tr>
<tr>
<td>ACT-PSA</td>
<td>0.019 µg/mg protein (41)</td>
<td>0.008 µg/mg protein (41)</td>
<td>Not detectable (40)</td>
</tr>
</tbody>
</table>
| cPSA           | 0.068 µg/mg protein (41) | 0.099 µg/mg protein (41) | ACT-PSA measurement could replace the tPSA test or both the fPSA and tPSA to further improve the discrimination between PCa and BPH (34). Thus far, controversial results regarding this assumption have been published and will be discussed separately.

#### Biological Explanation for the Different Occurrences of Serum PSA Forms in Patients with PCa and BPH

As of now, the reason for the quantitative differences in molecular forms between PCa and BPH in serum has not yet been clarified. Knowledge about the site of actual PSA complex forming at the molecular level would improve the interpretation of PSA values. Several hypothesis have been proposed to explain possible reasons.

(a) A higher ACT expression rate was found in PCa tissue, which could facilitate the complex formation between PSA and ACT at the cellular level (35). It was postulated that this increased intracellular complex formation would be responsible for the higher serum concentrations of ACT-PSA in PCa patients. Identical DNA sequence data of prostatic ACT in comparison with the hepatic ACT further corroborates the prostatic synthesis of ACT (36). Although another recent immunohistochemical study confirmed the presence of higher concentrations of ACT in PCa tissue than in BPH tissue (37), Igawa et al. (38) could not demonstrate a substantial amount of ACT-PSA complexes. It was hypothesized that the concentration of ACT in the prostate epithelium is not sufficient to bind the perhaps excess concentrations of PSA (40).

Recently, Jung et al. (41) analyzed the intracellular pattern...
of PSA forms by measuring fPSA, ACT-PSA, cPSA (Bayer Immuno 1 assay), and tPSA in prostatic tissue. The samples were obtained from cancerous and noncancerous parts of the same prostate, from adenomectomy specimens, and from transurethral resection material. fPSA was the main fraction in all tissue samples, whereas the complexed forms were <2% of tPSA. Two other research groups also found fPSA as the vast majority of intracellular PSA in quantitative measurements (42, 43). The amounts of fPSA and cPSA forms in benign and malignant prostatic tissue obviously do not explain the behavior of the fPSA:tPSA ratio in serum of PCa and BPH patients.

(b) Differential release of enzymatically active and inactive PSA from normal, BPH prostatic tissue and from prostatic cancerous tissue is another possible explanation for the higher serum concentration of serum ACT-PSA in PCa patients (44, 45). Because of the damaged basement membrane in prostate epithelial cells, PSA from PCa tissue can reach the circulation directly. In contrast, the PSA from normal or BPH tissue reaches the circulation much more slowly by leaking “backwards” into the extracellular space where it is susceptible to proteolytic degradation (45). Chen et al. (44) predicted a protease in BPH nodules that is probably responsible for the inactivation, or “nicking,” of PSA in BPH tissue. The inactive, or “nicked,” fPSA was isolated in BPH nodule fluids and in serum (44, 46). Such nicked PSA reacts with ACT in vitro poorly or not at all (47). A higher degree of nicked PSA in BPH may partially explain the smaller capability to form complexes with ACT.

(c) Recently proPSA forms in the serum and tissue of PCa patients were isolated (48, 49). proPSA was elevated in the peripheral-zone cancer but largely undetectable in the transition zone of matched sets, suggesting a closer correlation with PSA than with BPH (49). Whether the different distribution of proPSA in tissue of PCa and BPH is attributable to the different activation of proPSA to PSA or not remains to be elucidated.

Recently, the same research group identified a BPH-nodule-associated form of PSA called bPSA in BPH tissue (42). The median percent bPSA of PSA (tPSA was >99% of tissue PSA) was 11.4% in the transition zone of specimens with nodular BPH. In cancer tissue and in tissue without nodular BPH, the median percent bPSA values ranged significantly lower, from 3.2 to 4.9% (42). It may be possible that PSA in the serum of BPH patients contains a higher proportion of bPSA and results in a higher proportion of PSA in patients with BPH. Measurements of the cancer-associated proPSA together with the nodular BPH-associated bPSA may enhance further the ability to distinguish PCa from BPH (49).

(d) An irregular glycosylation process of PSA in dysplastic PCa cells was suggested to be the cause for a shift of the isoelectric points of PSA observed in the serum of PCa patients (50). The higher proportion of PSA forms with more basic pH in the serum of patients with PCa was discussed as a potential tool for a better distinction between PCa and BPH (50). Recently, this assumption gained new attention when PSA from a metastatic cell line showed glycosylation rates other than normal PSA (51).

 Altogether, the molecular heterogeneity of PSA concerning proforms (48, 49), nicked forms (44, 46), isoelectric point differences (50), various enzymatic activities (47), and different amounts of PSA fragments (52) forecast more research studies on measurements and clinical evaluations of these molecular forms of PSA (53). Modern methods of proteomics, including the techniques of two-dimensional electrophoresis (for protein separation) and peptide mass fingerprinting (for protein identification), may provide information of great value for functional assignments of PSA.

Clinical Validity of fPSA and tPSA%
Measurements of free PSA alone cannot add information in the absence of tPSA determination (19, 30). The diagnostic improvement will be evident, only when the ratio of fPSA to tPSA, also called fPSA%, is calculated (28 – 33). When fPSA values exceed 10 or 20 µg/liter, fPSA% does not contribute to the differentiation of PCa and benign diseases because the positive predictive value of fPSA alone may be as high as 50–85% (5). Thus, the role of fPSA% will be especially evident for values <10 µg/liter.

fPSA% as Discriminator between PCa and BPH in the “Gray Zone” of 4–10 µg/liter. The probability of PCa increases as fPSA% values decrease (16). In the gray zone of 4–10 µg/liter, fPSA% is used to improve the specificity, whereas for tPSA values <4 µg/liter, fPSA% may be useful to increase the sensitivity of PSA detection. Because most urologists recommend prostate biopsy in men with PSA serum concentration of >4 µg/liter, the fPSA% parameter is applied widely to decrease the number of biopsies with true-negative histological results and thus avoid unnecessary biopsies.

Numerous retrospective studies demonstrated that ~19–64% of unnecessary, negative biopsies could be eliminated (19, 28, 31, 32, 54, 55). Reasons for these substantial differences could be the use of a wide range of fPSA% cutoffs (14–28%) with different sensitivities from 78–95% and the different views of the urologists on when a biopsy would be necessary. A prospective, multicenter trial using a cutoff of <25% fPSA showed that unnecessary biopsies could be reduced by 20% while missing only 5% of PCa in the PSA range 4–10 µg/liter (29). For the same PSA range, using a cutoff of 20% fPSA, 29% of biopsies could be avoided (31). Another study using a 24% fPSA cutoff attained comparable results (56).

In our retrospective investigation on 233 PCa and BPH patients, we used a 22.5% fPSA cutoff at the 90%-sensitivity level (57). Compared with tPSA alone, the specificity increased from 18% to ~55% for tPSA values between 2 and 10 µg/liter. Using this cutoff, unnecessary biopsies could have been avoided in ~65% of BPH patients while only missing 8% of cancers (57).

fPSA% as an Early Indicator of PCa in the PSA Zone <4 µg/liter. About 20% of men with serum PSA levels between 2.6 and 4 µg/liter will have a clinically detectable PCa within a 3- to 5-year period (58, 59). On the basis of current results of the European Randomized Study of Screening for Prostate Cancer on 8621 men, Schröder et al. (60) calculated that nearly 50% of all detectable cancers can be diagnosed with PSA 0–4 µg/liter. Of 50 prostatectomy cases with PSA <4 µg/liter, 84% had a stage pT2 or less, but 48% had a Gleason score of ≥7.

According to the current understanding, these cancers must be considered aggressive but still organ-confined (60). In addition to the fact that 30–50% of men with PSA concentrations between 4 and 10 µg/liter already have extraprostatic disease at the time of surgery (5, 61), it is of utmost importance to identify these early nonpalpable cancers in a still-curable state.

For serum PSA concentrations of 2.5–4 µg/liter, Djavan et al. (62) compared PSA, PSA density, PSA transition zone density, PSA velocity, and fPSA% for early detection of PCa in a prospective evaluated population of 273 men. fPSA% was revealed to be superior to all other PSA indexes. At the 95%-sensitivity point, 29.3% of unnecessary biopsies could have been avoided (62). Vashi et al. (56) used a fPSA% cutoff of 0.1.
19% for a population of 41 men with tPSA values between 3 and 4 μg/liter to obtain a 90% sensitivity and to detect one cancer per 1.7 biopsies. For different tPSA ranges <4 μg/liter, Catalona et al. (63–65) also reported on various retrospective and prospective studies. In a population of 120 men with initial PSA levels between 2.8 and 4 μg/liter who had undergone biopsies, 7% were diagnosed with PCa (63). However, during the next 4 years, PCa was detected in an additional 15%. With a cutoff of 23% tPSA, the test could identify 93% of men with cancer and would avoid 28% of negative biopsies (63). Another study on 914 screening volunteers with a normal DRE and serum PSA levels between 2.6 and 4 μg/liter discovered 90% of all PCa at a 27% tPSA cutoff (64). The rate of 81% organ-confined tumors showed similarity to the above-mentioned findings by Schröder et al. (60), whereas a Gleason score ≥7 was examined in 11.8% of all cases (64) compared with 48% by Schröder et al. (60). Altogether, the additional usefulness of fPSA% for tPSA values <4 μg/liter is clearly visible, although two studies could not confirm these data (66, 67).

Factors Influencing fPSA%

Many studies have been performed to investigate physiological and pathological factors affecting fPSA (9). Significant changes have been verified regarding age, prostate volume, prostatic disease, or therapy. A better knowledge about potential factors influencing the fPSA% value would lead to a more accurate valuation and interpretation of this new calculated parameter.

tPSA. Data from various studies have described an inverse relationship between tPSA% and tPSA (reviewed in Ref. 8). Thus, higher tPSA levels are more commonly associated with lower fPSA% values and are often associated with advanced PCa. For the generally accepted tPSA ranges, where tPSA% offers the best chance for detecting PCa, a possible tPSA effect within 2–4 μg/liter or within 4–10 μg/liter should be considered as almost negligible. One recent study on patients with nonmalignant disease with a slightly larger tPSA range of 2.6–9.9 μg/liter revealed no correlation of fPSA% to tPSA (68). A more recent multicenter study on 229 cancer patients and 1480 noncancer patients clearly demonstrated a significant decrease of the mean fPSA% for the tPSA ranges from <4 μg/liter to 4–10 μg/liter and to >10 μg/liter in both patient groups (69). Additional studies are required to indicate whether a shift of the tPSA% cutoff for tPSA levels <4 μg/liter is practicable and useful.

Stage and Grade. Although tPSA directly correlates with tumor stage, this test cannot accurately predict the individual final pathological stage for a patient (70). Despite the demonstrated ability of tPSA% to improve the PCA detection rate at concentrations <10 μg/liter, reports of the utility of tPSA% for staging PCa has been controversial. Some authors described an inverse correlation of tPSA% to the pathological stage (71–73). Other studies noted no significant improvement in staging (74–76). A low tPSA% value appears to be associated with tumors of higher Gleason scores (73, 77, 78). Recently, a prospective, multicenter clinical trial study demonstrated that tPSA% followed by Gleason sum were the strongest predictors for the postoperative pathological outcome (79). This indicates a potential close relationship between both parameters.

Tumor Aggressiveness. A retrospective study on 1748 men yielded a very low risk to develop a PCa within 10 years if tPSA is <3 μg/liter and tPSA% is >18% (80). On the other side, the risk of having PCa is not negligible if the tPSA% is ≤18% despite a low tPSA of <3 μg/liter (80). A current study on 368 patients within the tPSA range of 2.51–4 μg/liter predicted a risk of 46% having PCa if the tPSA% is <10% (65).

In addition, lower tPSA% levels appear to be associated with unfavorable pathological findings such as larger tumors, higher Gleason scores, capsular penetration, and positive surgical margins (65, 78, 79). In a comparison of aggressive and nonaggressive PCas, it was demonstrated that the tPSA% values in patients with aggressive tumors were significantly lower 10 years before diagnosis, whereas tPSA did not show a difference at the time of diagnosis (81). Li et al. (77) confirmed the inverse relation of tPSA% to a higher aggressiveness of the histological grade. tPSA% appears to be a better predictor for tumor aggressiveness and pathological outcome than tPSA (71, 73, 78, 79, 81). These results emphasize the importance of tPSA% for potentially detecting unfavorable pathological findings in PCa even if the tPSA value looks inconspicuous.

PIN. High grade PIN is most likely a precursor of PCa and often is associated with this malignancy (82). An isolated high grade PIN leaves a 15-fold increase in the relative risk of an undiagnosed PCa (83, 84). Several studies were performed to answer the question of whether PIN affects the tPSA value in serum. Tarle et al. (85) found an intermediate mean value of tPSA% in patients with PIN (16.9%) compared with BPH (29.1%) and PCa (14.4%). However, 50% of patients with PIN were subsequently diagnosed with PCa, and the undetected concomitance of both diseases may have influenced the tPSA% value. Kilic et al. (86) evaluated mean decreasing tPSA% levels starting from BPH (31%) to low-grade PIN (25%), high-grade PIN (21%), and ultimately PCa (14%), but the difference between high-grade PIN and PCa was not significant. A multivariate analysis of sextant biopsies on 570 men showed no tPSA% differences between BPH (15.8%) and BPH associated with PIN (14.1%) and PCa (9.7%) compared with PCa coincident with PIN (11%), revealing no influence of PIN alone on tPSA% (87). Recently, the author confirmed these results on radical prostatectomy specimens (88). Ramos et al. (84) focused the investigation of patients with high-grade PIN and a longer negative follow-up for PCa to exclude a possible effect of concomitant PCa. They observed nearly similar tPSA% patterns for BPH and high-grade PIN and a significant difference between both patient groups to PCa patients.

Especially the latter three studies suggest that high-grade PIN has little influence on tPSA% (84, 87, 88). Because of the integrity of the basal cell layer in PIN tissue as opposed to PCa tissue, it can be assumed that PSA from PIN tissue will not take the same “direct” way into circulation, which probably leads to higher serum concentrations of cPSA in PCa patients (45). Therefore, it seems that an isolated PIN may not lower the tPSA% as would a PCa, so that a decreased tPSA% value should be always considered as evidence of PCa.

Chronic Prostatic Inflammation. Patients with chronic inflammation of the prostate show tPSA% values comparable with those of patients suffering from PCa (89). Ornstein et al. (68) found no difference in tPSA% between patients with acute inflammation of the prostate and nonmalignant patients for tPSA concentrations of 2.6–9.9 μg/liter. However, only 60 of 50 patients had no evidence of chronic inflammation, so its effect on tPSA% could not be evaluated (68). Recently, Morote et al. (90) also showed no influence of inflammation on tPSA and tPSA%, but only 23.2% of all patients were without inflammation. Contrarily, Scatttoni et al. (91) confirmed the results of Jung et al. (89). In particular, in the study by Scatttoni et al. (91), patients with an inactive form of prostatitis appeared to have a relatively higher risk of a fPSA% of <18%.

These divergent results make a final conclusion regarding
the common pathological diagnosis of a chronic inflammation and its impact on fPSA% difficult. However, the urologist should bear in mind this possibility of a false-positive result.

**Age.** It is generally accepted that there is a positive correlation between age and tPSA (31, 92–94). The fPSA also increases with increasing age, but the fPSA% remains unchanged (93). We have also observed that fPSA% was independent of age in 1160 healthy volunteers (95). Kalish *et al.* (94) confirmed the independence of fPSA% of age. In contrast, other recent studies have found a decrease of fPSA% with increasing age (96, 97) or an increase of fPSA% with mean patient age (31, 68, 69).

These conflicting results could be explained by the use of various assays (98) or by the biological variation of fPSA (99, 100). To date, a definitive assessment of the relationship of fPSA% to patients’ age is not possible.

**Race.** Comparisons of PCa patients have shown that tPSA concentration at diagnosis is higher in black men than in white men and that there is a higher incidence of more aggressive tumors in black men (101, 102). The probability of having PCa in black patients at tPSA 4–10 μg/liter is 30% compared with 25% in white patients (5). The objective of a recent multi-institutional study was to evaluate whether a selected fPSA% cutoff for white men yielded the same efficiency as in black men and whether fPSA% was useful in predicting pathological features in black patients, too (103). A total of 764 patients with tPSA values between 4 and 10 μg/liter, nunsuspicious DRE, and histologically confirmed diagnosis were enrolled. At the fPSA% cutoff of 25%, both races attained 95% sensitivity, whereas 20% of unnecessary biopsies could have been avoided in white men and 17% in black men. In both races, higher fPSA% values indicated a lower risk of PCa and also predicted a favorable pathological outcome (103). The apparent similarity in fPSA% results are promising for a possible race-independent prospective use. However, another recent study indicated that 32% of black men would not have been diagnosed with cancer at the 25%-fPSA% cutoff compared with only 13% of white men, even though the median fPSA% values did not differ between both races (104).

**Prostate Volume.** Catalona *et al.* (28) and Partin *et al.* (31) found an increase in fPSA% with increasing gland volumes. We observed a similar relationship for PCa, whereas fPSA% in nonmalignant patients was independent of prostate volume (105, 106). We evaluated the influence of different factors on fPSA% in 760 men (105). Prostate volume was found to have a remarkable impact on the ratio of fPSA:tPSA. If prostate volume does not exceed 40 ml, fPSA% can further enhance the ability to distinguish between BPH and PCa (106). More recently, Haese *et al.* (107) could confirm the diagnostic advantage of fPSA% for a gland volume of <60 ml. Ornstein *et al.* (68) found the positive relationship of fPSA% to prostate volume also in patients with only benign prostate diseases. To date, a multicenter study on 1709 men provided additional verification of the dependence of fPSA% on prostate volume in cancer and nonmalignant cases, demonstrating a significant positive correlation between fPSA% and gland volume (69).

### fPSA% as Helpful Tool for Prostate Biopsy

Most of the above-mentioned factors influencing fPSA% may enhance the clinical use and interpretation of the fPSA% value in PCa diagnosis. Presently, data on fPSA% indicate that for tPSA of 2–10 μg/liter and a normal DRE, the most helpful application of fPSA% is the indication for a biopsy or a repeat biopsy. Currently there are many different recommendations and different cutoffs for the use of fPSA% depending on the various assays used and on the targeted sensitivity or specificity (108). Within the tPSA range of 2–4 μg/liter, the fPSA% cutoff should yield a high specificity to avoid a great number of negative biopsies, but aggressive tumors should be detected below this cut-point, too. On the other hand, for tPSA levels >4 μg/liter, a 90% or 95% sensitivity is required to detect cancer in most patients.

Table 2 indicates our preliminary recommendation to perform a prostate biopsy depending on fPSA% values within one of the three different tPSA ranges measured with the IMMULITE tPSA and fPSA assays (109). For a repeat biopsy, we advise a 15% cutoff on fPSA within the gray zone (tPSA range 4–10 μg/liter) with an unsuspicious DRE. At this cut-point, the efficiency (e.g., sum of sensitivity and specificity) has the highest value. For tPSA concentrations between 2 and 4 μg/liter, a biopsy should be performed only if the fPSA% is <9%. However, aggressive cancer may be missed at this low fPSA% cutoff (65, 80). Thus, our preliminary decision criteria and recommendations (Table 2) should be considered as a starting point for other studies underway and for future studies. Additional prospective validations on larger cohorts are required for a definitive cutoff selection, especially for low tPSA levels (<4 μg/liter).

If prostate volume does not exceed 40 ml (Table 2), the cutoff of 15% fPSA also could be used as decision criterion for an initial biopsy in the tPSA range of 2–20 μg/liter because of a constant high efficiency level (106). Contrary to the proposed unrestricted use of one fPSA% cutoff in relatively small glands, fPSA measurements for enlarged glands should be handled with caution (106). The coexistence of BPH and PCa may render the differentiation between both diseases difficult. The question of whether a higher fPSA% cutoff or the use of PSA density or PSA transition zone density can increase the cancer detection rate in enlarged glands remains unanswered.

Because the urologists are using the fPSA% especially in patients with one negative biopsy to decide about further biopsies, the power of this marker has been discussed controversially. Stephan *et al.* (109) do not suggest a repeat biopsy if the fPSA% is >21%. In this group of patients, only 5% of cancers would be missed. Hayek *et al.* (110) detected PCa in 15.7% of all repeat biopsies, but concluded that no additional information was provided by fPSA% for a biopsy decision. In a recent large prospective study, ~10% of all initial negative biopsies were diagnosed with PCa in a repeat biopsy (111). In that study, fPSA% was the most accurate predictor of PCa in repeat biopsy specimens.

### Should fPSA% Be Used Generally in Screening for PCa?

Bangma *et al.* (112) reported in a screening study on 4800 men that additional fPSA measurements specifically for tPSA of

<table>
<thead>
<tr>
<th>fPSA% cutoff</th>
<th>tPSA range (μg/l)</th>
<th>Prostate volume (ml)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
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<tr>
<td>9</td>
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<tr>
<td>15*</td>
<td>2–20</td>
<td>≥40</td>
<td>60</td>
<td>68</td>
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*For further details see text (according to Refs. 106, 108, 109).*  
*Only limited diagnostic power.*
4–10 μg/liter were helpful. In contrast, Wald et al. (113) indicated no advantage in adding fPSA to tPSA in PCa screening trials. Because ~10% of all PSA measurements are in the tPSA zone of only 4–10 μg/liter, and 85% of all men have a PSA value < 4 μg/liter, a general additional use of fPSA in every tPSA determination seems ineffective. However, expanding the range of additional fPSA measurements from 4–10 μg/liter to 2–10 μg/liter could be beneficial for detecting more PCas at low tPSA values.

Additional Notes for Accurate Interpretation of fPSA

Sample Stability. Serum sample handling and stability data are important for interpreting fPSA% values because most investigators use stored samples for retrospective analyses. Woodrum et al. (114) reported that fPSA in serum samples was less stable than tPSA (115). tPSA decreased ~1%/h of clotting time, 2–3%/day, and subsequently 60–90%/month at 4°C or 23°C, but only 0.9% and 0.4%/month if stored at −20°C or −70°C, respectively (114). On the basis of these data, it is recommended to process and store the samples at 4°C within 2–4 h after blood sampling. Storage for longer than 24 h should be done at −70°C (114). A storage for more than 2 years at −70°C also showed no decline in fPSA and tPSA samples (115). Similar data have been reported by other authors (116–118).

PSA Clearance Rates. A comprehensive summary of PSA clearance rates is described in a review by Stenman (45). Because it is known that PSA in serum occurs in different molecular forms, several studies were performed to measure the half-life of fPSA, cPSA, and tPSA after radical prostatectomy. Biphasic models were established for elimination kinetics of fPSA and tPSA. Regarding fPSA, the first fast phase is presumably a result of the rapid loss of fPSA with an average half-life of approximately 0.5–2 h, whereas the slower second phase has half-life periods between 13.9 and 22 h (119–121). The initial and rapid decrease of fPSA may also be a result of new complexes forming with ACT and A2M (122). However, other authors assumed a rapid extracellular redistribution or glomerular filtration of fPSA (119, 123).

Knowledge about the faster elimination rate of fPSA compared with ACT-PSA is helpful in explaining low fPSA% values after a temporary increase of tPSA after prostate manipulations (DRE, biopsy, or ultrasound) or acute prostate inflammation. Despite an initial PSA increase after DRE, mainly attributable to a fPSA increase (124), those relatively higher fPSA concentrations are eliminated faster than ACT-PSA and result in changing fPSA% values. Therefore, it is useful to wait 4–6 weeks after such an event before measuring fPSA and tPSA.

Surgical and Drug Treatment History. Meyer et al. (105) investigated a possible effect of fPSA% after transurethral resection of the prostate. Compared with untreated BPH patients, the tPSA and fPSA levels were significantly decreased in patients treated by transurethral resection, but fPSA% did not differ between the two groups. In the serum of relapsing PCa patients after radical prostatectomy, the fPSA% varies within a relatively wide range and obviously does not give additional information (125).

Whereas Terazosin showed no effect on fPSA (126), treatment of BPH with 5α-reductase inhibitor finasteride decreased tPSA by ~50% in men without PCa (16). Despite the lower tPSA level, fPSA% did not significantly change in relation to untreated patients (105, 126–128). Although these studies suggest a possible continuous use of fPSA% for detecting PCa in treated BPH patients, additional prospective clinical trials are necessary to support these findings.

PSA Complexes

ACT-PSA. Most of the PSA in serum exists as a complex with ACT. The proportion of this complex is higher in PCa patients than in those with BPH (19, 21, 129, 130). ACT-PSA directly correlates with tPSA concentrations (34). It has been suggested that the higher proportion of ACT-PSA offers a better tool for detecting PCa compared with PSA (34, 130). There was the hope that the measurement of the ACT-PSA complex alone, instead of fPSA and tPSA determination, would additionally resolve the problems of different analytical conditions, e.g., calibration for fPSA and tPSA.

However, several analytical difficulties, such as the loss of immunoreactivity, complex dissociation during long-term storage, or the overrecovery caused by the presence of ACT-cathepsin G complex, impaired accurate ACT-PSA measurement (131–134). All these early technical problems in ACT-PSA determination explain why fPSA and the fPSA/tPSA ratio, and not ACT-PSA, were introduced first into clinical practice. Recently, the use of monoclonal antibodies specifically against ACT-PSA with low cross-reactivities to the ACT-cathepsin G complex, ACT and fPSA (135), the addition of heparin in the reaction mixture (133), and the application of special blocking reagents to reduce the nonspecific binding of anti-ACT antibodies (134) have been recommended to improve the analytical performance of ACT-PSA measurements.

Because of the analytical problems mentioned above, the results of ACT-PSA determinations are conflicting and are discussed controversially (19, 134, 136–138). Some authors recommend the measurement of ACT-PSA and the calculation of the ACT-PSA/tPSA ratio instead of fPSA% in view of a better differentiation between PCa and BPH (19, 21, 134, 138). In addition to the advantageous use of ACT-PSA to fPSA%, Espana et al. (137) also provided higher specificity using citrated plasma instead of serum. Using a prototype assay for ACT-PSA in serum, we did not find enhanced utility for ACT-PSA and the calculated derivatives for differentiation between PCa and BPH compared with fPSA% (139). As shown in Fig. 1, fPSA% had the greatest area under the ROC curve followed by the fPSA:A2M:PSA ratio, ACT-PSA, tPSA, and the ACT-PSA:ACT-cathepsin G complex. tPSA% offered the highest efficiency within the tPSA range of 2–20 μg/liter to discriminate between cancer and non-cancer patients (139). These data are consistent with results from other investigators (136, 140–142). A more recent multicenter study of 657 men for PSAs of 2–20 μg/liter confirmed strongly that ACT-PSA alone was not better than fPSA%, and the ratio of ACT-PSA to tPSA corresponds to the diagnostic power of fPSA%. 5

cPSA. A novel PSA assay that measures all cPSAs except the PSA-A2M complex has been introduced recently (143). This assay, which is now available from Bayer Diagnostics, is based on the unique binding properties of the capture monoclonal antibody MM1. This antibody fails to bind fPSA in the presence of a second specific monoclonal antibody (ME2) against epitope E, the specific epitope for fPSA. Preincubation with an excess of ME2 reduces the reactivity of fPSA to <3%, and thereafter the MM1 antibody detects all PSA complexes but not

fPSA (143). Both the predominant ACT-PSA as well as the API-PSA are detected in this complex PSA assay (143, 144). The A2M-PSA complex is not detected (144).

Using this newly developed assay, Brawer et al. (145) reported that cPSA alone was a better discriminator between PCa and BPH than tPSA or fPSA% in the tPSA range of 4–10 μg/liter. In a cohort of 36 PCa patients and 117 patients with no evidence of malignancy, the cPSA showed the greatest area under the ROC curve (0.73). Moreover, tPSA was also better than fPSA% (areas under the ROC curves were 0.66 versus 0.6). This contradicts several studies showing that fPSA% significantly enhances the specificity for tPSA values >10 μg/liter (28, 29, 31, 32, 54, 55). The findings of lower values for fPSA% compared with tPSA in the area under the ROC curve indicate inappropriate cohorts (144).

Jung et al. (57) could not show an advantage of cPSA alone at tPSAs of 2–10 μg/liter in reducing the rate of unnecessary biopsies (see Fig. 2A and B). On the other hand, the ratios fPSA:tPSA and cPSA:tPSA, both comparable, improved the differentiation between PCa and BPH related to tPSA (57). Similarly to fPSA%, the ratio cPSA:tPSA can significantly enhance the specificity and thus reduce the rate of inappropriate prostate biopsies. Meanwhile, other research groups confirmed that the ratio of fPSA:tPSA is still superior to cPSA alone (135, 146).

The suggestions by Brawer et al. (145, 147) and Maeda et al. (148) that cPSA can replace tPSA or both tPSA and fPSA need additional studies. Reducing the costs of two measurements (fPSA and tPSA) and avoiding a possible quotient bias or manufacturer’s assay variability appear promising for the use of cPSA alone. However, both practical experience by several investigators and the theoretical background do not support such optimistic expectations (144, 149). Filella et al. (146) observed that cPSA was only more useful than tPSA in patients with tPSA concentrations between 3.1 and 6 μg/liter. The maximum capacity for the correct reclassification of BPH patients was obtained when tPSA values were between 4.1 and 5 μg/liter.

Finally, it should be noted that the cPSA assay also measures the relatively small amounts of API-PSA complex, which is more associated with benign disease compared with the main complex of ACT-PSA, which is higher in PCa (144, 150). This could lead to a diagnostic uncertainty in interpreting the cPSA value. Furthermore, it cannot be excluded that the capture monoclonal antibody MM1 still detects fPSA despite blocking the fPSA epitope E because of different fPSA epitope-recognition of MM1 and ME2 (143). This could lead to a slight overestimation of cPSA.

Despite the limiting factors for cPSA, the analytical reliability is superior to ACT-PSA. Measurements of both tPSA and cPSA have displayed a comparable utility to tPSA and fPSA (57, 146). Regarding the analytical advantage of cPSA compared with fPSA, because of the greater serum concentration and the presumed higher stability of cPSA, it cannot be
ruled out that the percentage of cPSA may be prospectively more useful than fPSA%. Additional studies are required to compare fPSA% and the cPSA:PSA ratio to answer this question.

A2M-PSA. Since the middle of the last decade, different research groups have investigated the immunological detection of the A2M-PSA complex (25, 47, 151, 152). Under in vivo and in vitro conditions, PSA was found to bind more rapidly and more aggressively to A2M than to ACT (25, 47, 151). An early immunoassay partially measured A2M-PSA only at high tPSA levels with an insufficient detection limit of 3 μg/liter (152). Overall, the main difficulty was the encapsulation of PSA because of A2M and the subsequent loss of PSA immunoreactive epitopes. Recently, Zhang et al. (27) described an assay with an acceptable detection limit of 0.14 μg/liter. Their method is based on removal of all immunoreactive PSA from serum by immunoadsorption and release of the encapsulated PSA from A2M by pH variation. At a 30%-PSA recovery rate of the A2M-PSA, the median ratios of A2M-PSA:PSA were 12% for PCa patients and 17% in BPH patients.

ACT-PSA and A2M-PSA complexes bind to the A2M receptor/low-density lipoprotein receptor, which may be the clearance receptor for PSA (153). Kinetic analysis revealed faster binding of PSA to A2M than to ACT, confirming earlier suggestions. Because of the extremely short half-life of A2M and the rapid binding of PSA to A2M, it was hypothesized that common measurements of PSA incorrectly calculate the PSA concentrations when released from the prostate into circulation (153). Using radiolabeled PSA injected into rats, Birkenmeier et al. (154) were able to demonstrate recently that A2M-PSA is solely eliminated by the liver, whereas ACT-PSA is eliminated by the liver and by the kidney. Thus, factors that modulate the elimination receptors or organs may interfere with the steady-state concentration of the different molecular forms of PSA, keeping in mind that A2M is the main inhibitor of PSA in blood. It has been suggested by Stenman that the sum of A2M-PSA and PSA in serum might reflect the complete or real fPSA fraction in vivo because of in vitro complex formation between PSA and A2M after sampling (155). The parallelism of higher frPSA and higher A2M-PSA levels in BPH patients may further improve differentiation between BPH and PCa.

In contrast with the measured A2M-PSA values up to 49 μg/liter by Zhang et al. (27), another report by Lilja et al. (123) could not confirm these high concentrations of A2M-PSA. Lilja et al. (123) developed an assay with a detection limit of 2 μg/liter. Only 1 of 18 PCa patients had a measurable A2M-PSA concentration. After radical prostatectomy, almost all A2M-PSA concentrations were below the analytical detection limit.

As the analytical problems of the A2M-PSA measurements have not yet been resolved, estimation of the clinical validity of A2M-PSA remains difficult.

API-PSA. The development of highly sensitive immunoassays for detecting additional PSA complexes has gained more attention in the last years. To date, none of these tests are commercially available. After Zhang et al. (156) indicated that PSA slowly forms a complex with API in vitro, the same group developed an assay analogous to the ACT-PSA assay with an antibody specific for API (150). The differences in the median API-PSA:tPSA ratios were significant between PCa (3.2%) and BPH patients (4.1%). Despite the relatively low proportions of API-PSA to tPSA concentrations, these results warrant additional investigations on a possible utility of API-PSA as an adjunct to the other molecular forms of PSA in serum.

hK2
The KLK2 gene (encoding for hK2 protein) and the KLK3 gene (encoding for hK3 or PSA) are members of the human kallikrein family (24, 157). Together with the renal/pancreatic kallikrein (KLK1, encoding for hK1) and many other newly detected kallikrein genes (reviewed in Ref. 158), these 14 human kallikrein genes belong to the serine protease family. The renal/pancreatic kallikrein hK1 shares 62% homology with PSA (159), whereas hK2 shows 78% amino acid sequence identity to PSA (160). At the DNA level, hK2 and PSA share 80% similarity (161, 162). The secretion of hK2 is similarly to PSA stimulated by androgens, and it is mostly found in prostatic tissue. The mRNA concentration for hK2 amounts to 10 and 50% of the PSA mRNA concentration in the prostate gland (163, 164). Although the hK2 concentration in seminal plasma is only 0.1–1% of the PSA concentration, hK2 has the capacity, like PSA, to cleave the gel-forming proteins semenogelin I, semenogelin II, and fibronectin (165, 166). The hK2 has been purified and characterized from seminal plasma where most of the protein was found in complex with PCI (167).

hK2 manifests a trypsin-like substrate specificity (168, 169), whereas PSA has a restricted chymotrypsin-like activity (170). It has been suggested that the regulation of hK2 is inhibited by zinc (166). hK2 also forms complexes with various plasma protease inhibitors such as α2-antiplasmin, antithrombin III, C1-inactivator, plasminogen activator inhibitor-1, and A2M (171–173). All molecular forms of hK2 in prostatic tissue, seminal plasma, and serum or plasma are summarized in Table 1.

As opposed to PSA, hK2 can activate the zymogen form of urokinase (174). This shows that hK2 has potential plasmin-like activity and suggests that hK2 could be the initiator of a proteolytic cascade leading to prostatic cancer invasion (174). In 1997, hK2 had been shown to cleave proPSA to generate enzymatically active PSA (169, 175, 176). This suggests that hK2 may play a physiological role in the regulation of PSA activity. Hence, it has been presumed that these two molecules also might act in concert in extraprostatic locations. Until now, PSA and hK2 have been found and measured together in biological fluids and tissues, e.g., in amniotic fluid, breast milk, breast cyst fluid, and in malignant and nonmalignant breast tissues. In other tissues, such as in pituitary tissues and human endometrium, only the mRNAs for hK2 and PSA have been detected (177–183). The hK2 concentrations were always approximately two orders of magnitude lower than PSA or hK2 in serum and seminal plasma.

hK2 was also detected in the urine. Like PSA in urine, it is not clinically useful for early detection or staging of PCa (184), but both proteins are down-regulated in urine and plasma after antiandrogen treatment (185).

hK2 in Prostatic Tissue. Initial immunohistochemical studies revealed differences in the expression of hK2 and PSA. PSA immunoreactivity was most intensive in benign epithelium and stained to a lesser extent in PIN and to the least extent in prostate carcinoma. In contrast, hK2 intensity and extent were greatest in cancer with the most intense staining in the highest grades of cancer with less intense staining by PIN and the lowest in benign epithelium (186, 187). Darson et al. (188) demonstrated in 151 radical prostatectomy patients with lymph node-positive PCa a higher expression of hK2 in lymph node metastases than in primary cancer. Furthermore, the hK2 expression in primary cancer was greater than in benign epithelium (188). prohK2 was expressed in a greater percentage of cells in primary cancer than in benign tissue. In contrast to hK2, prohK2 and PSA were expressed to a greater extent in primary
PCa than in lymph node metastases. Consequently, the authors assumed that the prostate tissue expression of hK2 appears to be regulated independently of PSA (188).

Magklara et al. (189) measured hK2 and PSA concentrations in paired cancerous and noncancerous prostatic tissue using sensitive and specific immunofluorometric procedures. Similar to the relations of PSA and hK2 in seminal plasma and male serum, hK2 only presents 1–2% of the PSA concentration in prostate tissue. Both prostate kallikreins are expressed more in noncancerous than in cancerous tissue, whereby the degree of down-regulation was higher for PSA than for hK2 (189). These findings are in contradiction to previous data generated by immunohistochemistry (186, 188). A recent study on aspiration biopsy material showed that the down-regulation of PSA in PCa patients can predict the outcome of endocrine treatments better than clinical classifications such as tumor stage and cytological grade (190). Thus, the physiological mechanism of the intracellular PSA and hK2 regulation requires additional investigation.

Recently, a novel hK2 complex was identified in prostate tissue consisting of hK2 and a serine protease inhibitor known as PI-6 (191). This complex represents about 10% of thK2 in the tissue and is increased in the tumor tissue. Interestingly, no comparable complex of PSA and PI-6 was detected (191). Whether this PI-6-hK2 complex itself is variable in tumor tissue compared with benign tissue or correlates with early neoplastic development should be investigated further.

**hK2 in Serum.** As already mentioned, serum concentrations of hK2 are usually <3% of the PSA concentrations (192, 193). This finding verified the early analytical difficulties for hK2 immunoassays, because lower detection limits were above the biological concentrations of hK2 in serum. The first assays had an analytical detection limit (signal imprecision + 2 SD of the zero calibrator) of 100 ng/liter (193). The assays of the recent generation have an analytical detection limit of <10 ng/liter (192, 194–196). However, clearly different concentrations in healthy men have been measured (192, 196). The reasons for these discrepancies were not known until now (197).

Analogous to PSA, hK2 in serum exists in different molecular forms (193, 198). Free hK2 is the most abundant form in serum (198), whereas ACT-hK2 represents only 4–19% of thK2 (195). The zymogen form of hK2, prohK2, is also present in serum (198), whereas ACT-hK2 represents only 4–19% of thK2 (195). The zymogen form of hK2, prohK2, is also present in human sera and increased in prostate diseases (199). Although very sensitive analytical hK2 assays are essential for studies of serum hK2, equimolar tests may not be as important as for PSA because of the relatively low proportions of complexed hK2 in serum.

Klee et al. (192) developed an ultrasensitive automated assay for hK2 with a detection limit of 1.5 ng/liter. The median serum concentration of hK2 was 26 ng/liter in healthy men. PCa patients with lower Gleason scores had a median hK2 concentration of 72 ng/liter compared with 116 ng/liter in patients with a more advanced cancer (192). A recent study by Becker et al. (200) indicated that increased hK2 concentrations are associated with an increase of aggressive PCa. Furthermore, for differentiation between BPH and PCa patients, the calculated ratio hK2/tPSA significantly enhanced the specificity at the 95% sensitivity level (200).
median hK2 of 135 ng/liter in PCa patients and a median hK2 of 90 ng/liter in BPH patients (P < 0.1). The ratio of hK2:fPSA showed the highest significance in discriminating PCa and BPH, whereas the ratio hK2:tPSA could not increase the efficiency compared with the ratio fPSA:tPSA (194). Later, these findings were established on a larger patient population (201). A study on 937 archived serum samples by Partin et al. (202) for tPSA concentrations of 2–10 μg/liter demonstrated an advantage in combining the fPSA% and the hK2:tPSA ratio for detecting PCa. Regarding the fPSA% values within the tPSA range of 2–4 μg/liter, the additional use of hK2:tPSA would identify as many as 40% of the cancers and would require biopsy in only 16.5% of the men with these low-tPSA concentrations (202). Magklara et al. (203) studied the effect of the three-parameter fPSA, tPSA, and hK2 on 206 PCa and BPH patients at tPSAs of 2.5–10 μg/liter. hK2 alone was not shown to distinguish between PCa and BPH (203, 204). Again, only the ratio hK2:tPSA provides additional information. The authors suggest prostate biopsy if the hK2:tPSA ratio is >1 within the tPSA range of 2.5–4.5 μg/liter because of a 35% likelihood of having cancer (203). A study by Nam et al. (204) among men referred for prostate biopsy illustrated a 5- to 8-fold increase in risk for PCa at high hK2 levels, adjusted to PSA and other risk factors. The results of two recent studies suggest a significant increase of hK2 concentrations with increasing grading levels, especially for non-organ-confined cancers (205, 206).

These preliminary studies on the diagnostic validity of hK2 already demonstrate the potential usefulness of the new marker hK2 as a valuable adjunct to the established PSA values and multivariate analyses (207, 208) for an improved differentiation between PCa and BPH. However, more work has to be done to make this test ready to use in clinical practice. Two points have priority: (a) larger multicenter studies are essential to prove the clinical usefulness of hK2 in comparison with conventional parameters in prostate carcinoma diagnostics; and (b) technical problems to measure the different molecular forms of hK2 using appropriate antibodies have to be solved. In particular, the recent report on an accurate and specific assay for complexed hK2 may provide additional enhanced specificity (209).

Conclusion and Future Trends

Table 3 outlines the current clinical utility of the molecular forms of PSA and hK2 as indicators for PCa diagnosis. To date, measurements of the molecular forms of PSA allow a better prognosis of having PCa for tPSA values up to 20 μg/liter. This significantly reduces the number of unnecessary biopsies.

fPSA measurements and the use of its ratio to tPSA are well established in numerous, large, multi-institutional clinical trials. A forthcoming standardization of the fPSA and tPSA measurements will provide a better comparability of different PSA assays. In the near future, the fPSA% value will be used more frequently with additional clinical and laboratory values to calculate the risk of having cancer. Algorithms like artificial neural networks and various logistic regression models could give higher predictive values for finding malignancy on a biopsy, and this appears to be the most effective way of obtaining an accurate diagnosis of PCa. However, prospective studies have to show that the decision of urologists to decrease the biopsy rate on the basis of fPSA% does not have a negative effect on the safety of the patients. Thus, studies assessing the outcome of populations of men managed on fPSA% values are needed.

The use of the other molecular forms of PSA and hK2 offers interesting and promising approaches for additional improvements in differentiating BPH from PCa. When the analytical problems are resolved, these parameters should be evaluated in clinical trials to test the possibility of adding information in PCa detection. We expect rapid progress in the whole area of PCa research within the next few years.

In conclusion, we predict that better insight in regard to PSA and hK2 molecular forms will lead not only to better diagnostic markers, but also to an understanding of PCa development and possible new therapeutic options.

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