

# The Role of Molecular Forms of Prostate-Specific Antigen (PSA or hK3) and of Human Glandular Kallikrein 2 (hK2) in the Diagnosis and Monitoring of Prostate Cancer and in Extra-Prostatic Disease

*Charlotte Becker,<sup>1</sup> \* Joachim Noldus,<sup>2</sup> Eleftherios Diamandis,<sup>3</sup> and Hans Lilja<sup>1</sup>*

<sup>1</sup>Dept. of Clinical Chemistry, Lund University, Malmö University Hospital, Malmö, Sweden; <sup>2</sup>Dept. of Urology, University of Hamburg, Hamburg, Germany; <sup>3</sup>Dept. of Pathology and Laboratory Medicine, Mount Sinai Hospital, University of Toronto, Toronto, Ontario, Canada

**Referee:** H. Rittenhouse, Hybritech, 7330 Carroll Road, San Diego, CA 92121, USA

\* Corresponding author: Charlotte Becker, M.D. Ph.D., Dept. of Clinical Chemistry, Lund University, Malmö University Hospital, 205 02 Malmö, Sweden. Tel: +46-40-33 22 27. Fax: +46-40-33 70 43. e-mail: [charlotte.becker@klkemi.mas.lu.se](mailto:charlotte.becker@klkemi.mas.lu.se)

**ABSTRACT:** Prostate-specific antigen (PSA or hK3) is a glandular kallikrein with abundant expression in the prostate that is widely used to detect and monitor prostate cancer (PCa), although the serum level is frequently elevated also in benign and inflammatory prostatic diseases. PSA testing is useful for early detection of localized PCa and for the detection of disease recurrence after treatment. However, PSA has failed to accurately estimate cancer volume and preoperative staging. There is no PSA level in serum that definitively distinguishes men with benign conditions from those with prostate cancer, although PCa is rare in men with PSA levels in serum < 2.0 ng/ml. This prompted searches for enhancing parameters to combine with PSA testing, such as PSA density, PSA velocity, and age-specific reference ranges. Due to the protease structure, PSA occurs in different molecular forms in serum and their concentrations vary according to the type of prostatic disease. Human glandular kallikrein 2 (hK2) is very similar to PSA, but expressed at higher levels in prostate adenocarcinoma than in normal prostate epithelium. Blood testing for hK2 combined with different PSA forms improves discrimination of men with benign prostatic disease from those with prostate cancer. Many data have also been reported on the extra-prostatic expression of both PSA and hK2, and it is now believed that they may both have functions in tissues outside the prostate.

**KEY WORDS:** carcinoma, tumor markers, human glandular kallikreins, breast cancer, free PSA, complexed PSA.

## I. INTRODUCTION

The traditional human glandular kallikrein family, a subgroup of the serine protease family, comprises three proteins that are very similar in structure: hK1, or tissue kallikrein; hK2, or human glandular kallikrein 2; and hK3, which is usually designated prostate-specific antigen (PSA) due to its very high expression in the prostate. hK2 has 66% identity in primary structure to tissue kallikrein and 79% identity to PSA.<sup>1</sup> The genes encoding these proteins (KLK1, KLK2, and KLK3) are located close to each other in a 60- to 70-kb gene segment on the long arm of chromosome 19.<sup>2-4</sup> Expression of hK1 is mainly found in the salivary glands, the pancreas, and the kidneys.<sup>5</sup> It catalyzes the release of Lys-bradykinin from low- or high-molecular-weight kininogen and thus is involved in the regulation of blood pressure and local blood flow, as well as in inflammation and cell proliferation.<sup>6</sup> The highest expression levels of both PSA and hK2 are found in the prostate epithelium.<sup>7-9</sup> Recently, Diamandis *et al.*<sup>10</sup> reported that the human kallikrein gene locus is much larger, spanning about 300 kb, and includes 12 additional genes. All these genes encode putative serine proteases with a conserved catalytic triad (His, Asp, Ser). At the DNA and amino acid levels they show 30 to 80% sequence homology. Steroid hormones regulate many of these genes. Moreover, some have an expression level that is downregulated in breast cancer and some of the gene products appear to act as tumor suppressors. At present, little is known about the biological function of the protein products of the 12 additional genes. Two of the newly detected genes, prostase/KLK-L1/KLK4 and KLK 15, also show primarily prostatic tissue expression.<sup>11,12</sup>

## II. PROSTATE-SPECIFIC ANTIGEN

### A. Biochemical and Physiological Characteristics of Prostate-Specific Antigen (PSA)

PSA was first isolated from seminal plasma in 1971 by Hara *et al.*<sup>13</sup> In 1979, Wang *et al.*<sup>14</sup> purified PSA from human prostate tissue and found it to be “prostate specific”. Shortly after, researchers detected PSA in human serum and suggested it as a marker of PCa.<sup>15-17</sup> PSA mRNA codes for a 261 amino acid prepro form of the protein, although the mature, catalytically active, single-chain form of PSA contains 237 amino acid residues with a single carbohydrate side chain attached at Asn<sub>45</sub>.<sup>18-20</sup> The molecular mass, including the carbohydrate side chain, is 28.4 kDa.<sup>21</sup> SDS-PAGE has indicated masses of approximately 33 and 28 kDa for the reduced and the nonreduced protein,

respectively, a difference explained by the internal disulfide bonding of the polypeptide chain. Processing of the 261 amino acid protein involves two steps: removal of the signal peptide after transfer of the protein to the ER, and removal of propeptide after activation of the protein.<sup>18</sup> The final step involving conversion of the zymogen form of PSA (proPSA) into the enzymatically active mature protein can be accomplished *in vitro* by adding hK2 at physiological ratios to proPSA.<sup>22-24</sup> However, also trypsin and now very recently prostin have been shown to convert proPSA into active PSA,<sup>24,25</sup> and it is not yet fully established how the conversion of proPSA to the mature, active 237 amino acid single-chain enzyme is accomplished and regulated *in vivo*.

PSA is a serine protease with chymotrypsin-like substrate specificity that mainly cleaves peptide bonds on the C-terminal side of certain tyrosine and leucine residues.<sup>26-29</sup> PSA is expressed by normal human prostate epithelium, epithelial cells in benign hyperplastic nodules, and a majority of the tumor cells in most prostate cancers.<sup>14</sup> PSA is secreted into seminal fluid, where it occurs at very high concentrations, 0.4 to 3 g/l, which corresponds to about 1.5 to 100  $\mu\text{mol/l}$ .<sup>16,30,31</sup> Here PSA degrades the gel-forming proteins semenogelin I and II and fibronectin that results in the liquefaction of semen and release of progressively motile spermatozoa.<sup>32-37</sup> Several reports have also suggested that PSA modifies cell growth. Studies *in vitro* have shown that the protease activity can modify insulin-like growth-factor-binding protein 3 (IGFBP3), which results in decreased binding affinity for IGF-1,<sup>38</sup> and PSA can inactivate the parathyroid-hormone-related protein (PTHrP),<sup>39</sup> although the significance of these effects *in vivo* is not clear. Moreover, PSA has been shown to stimulate the mitogenic activity of osteoblasts, possibly through activation of transforming growth factor- $\beta$  and proteolytic modification of cell-adhesion receptors.<sup>40</sup> PSA has also been reported to exhibit antiangiogenic activity.<sup>41</sup>

In the various extracellular compartments, catalytically active 237 amino acid single chain PSA can be inactivated by several major extracellular proteinase inhibitors, such as alpha-1-antichymotrypsin (ACT), alpha-1-proteinase inhibitor (API, also called alpha-1-antitrypsin or AAT), protein C inhibitor (PCI), alpha-2-macroglobulin (AMG), and pregnancy zone protein (PZP).<sup>28,42-48</sup> Several of these inhibitors that normally are present at levels of 0.5 to 30  $\mu\text{mol/l}$  in the blood therefore occur at hundred- to thousand-fold molar excess to PSA in the blood. By contrast, the majority, about 60% to 70% of PSA in seminal fluid, appears to occur in catalytically active form,<sup>28,33</sup> whereas not more than approximately 5% of PSA in seminal fluid exists as proteinase inhibitor complexes. PSA is mainly linked as covalent 1:1 molar ratio complexes with PCI,<sup>45</sup> which is mainly contributed by the seminal vesicles. The remaining 30% to 40% of catalytically inactive PSA in seminal fluid exists mainly as internally cleaved, two-chain or multichain forms of the

protein,<sup>28,49</sup> reported to be the result of cleavages C-terminal of mainly Lys<sub>145</sub>, but also cleavages C-terminal of Lys<sub>182</sub>,<sup>28,49,50</sup> although the modifying protease(s) responsible for these cleavages has not yet been clarified.

## B. Characteristics of PSA in Blood

The majority of PSA found in blood is catalytically inactive, mainly due to the formation of covalent 1:1 molar PSA-ACT complexes.<sup>42,43</sup> *In vitro*, PSA also rapidly forms complexes in the blood with AMG.<sup>42,43,46,51,52</sup> The complex formation with AMG, contrary to the mechanisms for complex formation with ACT, only blocks access to the catalytic cleft of PSA for large-sized protein substrates but not for small-sized peptide substrates. However, this PSA-AMG complex is difficult to measure, both because it is suggested to be present only at very low levels *in vivo*, but also due to the steric conformation of the AMG molecule that subsequent to the protease attack blocks access to the PSA epitopes. A recent study of the metabolism of different molecular forms of PSA after radical prostatectomy showed that immediately after surgery the level of free PSA was elevated almost tenfold,<sup>52</sup> but there was no significant increase in PSA-ACT or PSA-AMG concentrations. This indicates that the free PSA released into blood by prostatic manipulation during removal of clinically localized tumors is not eliminated by complex formation with the inhibitors in the blood *in vivo*, and thus suggests that the released free PSA-form is enzymatically inactive. However, PSA-AMG complexes are readily generated during nonoptimized storage of blood *in vitro*.<sup>51,52</sup> Low concentrations of PSA in complex with API have also been detected in blood.<sup>48</sup> A minority, 5% to 45%, of the immunodetectable PSA in blood is found in free noncomplexed form(s). The free noncomplexed PSA is most likely catalytically inactive as it remains unreactive (or only very slowly reacts) with the very large excess of active inhibitors such as ACT and AMG in the blood.<sup>53,54</sup> Several recent reports suggest that the composition of free PSA in the blood manifests considerable structural heterogeneity. Some findings suggest that the free PSA form in blood is mainly composed of unclipped inactive forms of PSA, including different proPSA forms,<sup>55</sup> whereas other reports suggest that it consists of internally cleaved PSA forms (also called “nicked” PSA).<sup>56</sup> More data on the free forms of PSA occurring in blood might soon be reported. A recent publication reports on the generation of monoclonal antibodies raised against a neo-epitope formed after internal cleavage of PSA at Lys<sub>182</sub>-Ser<sub>183</sub> (also called BPSA)<sup>57</sup> as well as monoclonal antibodies that only bind to the single-chain PSA forms (i.e., different proPSA forms and mature PSA).<sup>58</sup>

The first data reported on the half-life of PSA in serum by Stamey *et al.*<sup>59</sup> suggested it to be  $2.2 \pm 0.8$  days, whereas data from Oesterling *et al.*<sup>60</sup> suggested  $3.2 \pm 0.1$  days. Later, Partin *et al.*<sup>61</sup> reported that the clearance of free and total PSA after radical prostatectomy followed a “two compartment” model. The initial half-lives were  $<2$  h for free and total PSA and then increased to 22 and 33 h respectively. Björk and colleagues<sup>62</sup> also analyzed serum collected immediately after prostatectomy and follow-up samples collected up to 14 days. They reported only very slow, capacity-limited linear decline in the 95-kDa-sized PSA-ACT complex levels in serum, with a mean decrease of 0.8 ng/ml per day. By contrast the 28.4-kDa sized free PSA was eliminated in a bi-exponential manner, where initial re-equilibration gave a half-life of 0.8 h, and a second half-life of 14 h that might be explained by renal glomerular filtration.<sup>62</sup> Studying rats, Birkenmeyer *et al.*<sup>63</sup> observed that the PSA-AMG complex was cleared very rapidly from the circulation, with a calculated half-life of  $6.7 \pm 1$  min, which would explain the very low concentrations of this complex found in serum.<sup>51</sup>

One reason for the varying rates of elimination reported for the different molecular forms of PSA might be that this process occurs by several different pathways. The smaller sized free PSA molecule is probably subject to renal clearance, whereas the PSA-ACT and PSA-AMG complexes are too large to allow elimination by the renal glomeruli. Therefore, it is assumed that PSA-ACT and PSA-AMG may both be eliminated mainly by the liver, although by different mechanisms. In rats, Birkenmeyer *et al.*<sup>63</sup> showed that the PSA-AMG complex was metabolized in the liver, whereas the PSA-ACT complex was metabolized in both the liver and the kidneys. These researchers also noted that the rate of elimination of the PSA-AMG complex was considerably prolonged after addition of a 1000-fold molar excess of transformed AMG, which suggests that clearance of this complex is mediated via the AMG receptor in the liver. Furthermore, Birkenmeyer and co-workers observed that an excess of transformed AMG did not interfere with the clearance of the PSA-ACT complex in rats. This may have occurred because the PSA-ACT complex binds to a different site on the AMG receptor than AMG,<sup>47</sup> or more likely that it binds to another receptor in the liver and perhaps also in the kidneys. It has also been reported that ACT complexes do not bind to the AMG receptor,<sup>64</sup> whereas they bind to both the mouse serpin receptor in the liver,<sup>65,66</sup> and the rat glycoprotein gp 330 receptor in the kidneys.<sup>67,68</sup>

### **C. Clinical Applications**

The production of PSA is androgen dependent in the normal prostate gland and PSA can therefore be detected after puberty in the serum of healthy men.

The concentration of PSA is normally very low in serum ( $\leq 2.0$  ng/ml in men 40 to 49 years old), and it increases not only in PCa but also in benign prostatic hypertrophy (BPH),<sup>69</sup> hence many attempts have been made to establish appropriate reference ranges for serum PSA. A PSA cut-off of 4.0 ng/ml has been used most often over the years.<sup>69</sup> Oesterling reported that if this cut-off level were used as the decision level for biopsy in a group of men with organ-confined tumors or BPH, a sensitivity of 57% and a specificity of 68% would be obtained, and the positive predictive value (PPV) for serum PSA would be 49%.<sup>69</sup> Catalona *et al.*<sup>70</sup> reported that in a large screening study comprising 6630 males 50 years or older, 15% had a PSA concentration greater than 4.0 ng/ml. After biopsy of these men, the PPV for PSA was 32%. Numerous other studies show more or less equivalent data, which clearly indicate that many cancers will be missed with a PSA cut-off of 4.0 ng/ml. In one study Catalona *et al.*<sup>71</sup> found cancer in 22% of men with PSA concentrations of 2.6 to 4.0 ng/ml who underwent biopsy. Accordingly, lowering the cut-off level would result in better sensitivity, but this would also reduce the specificity and PPV. This low specificity is a problem when using PSA as a tumor marker, and many attempts have been made to improve the specificity of PSA measurements.

Oesterling and co-workers<sup>72</sup> evaluated appropriate reference ranges in serum from a randomly chosen population of healthy men aged 40 to 79 years and living in Olmsted county, Minnesota. This study showed that age as well as prostate volume correlated with serum PSA concentrations, thus the use of age-specific reference ranges was introduced. However, even though age-specific reference ranges would lead to enhanced sensitivity for PCa in men younger than 60 years and enhanced specificity in men older than 60 years, these ranges have been criticized because they would also miss cancers in men older than 60 years, a group at highest risk for PCa.<sup>73,74</sup>

To improve the specificity of PSA measurements, Carter *et al.*<sup>75</sup> used another approach in which the PSA velocity was analyzed by serial measurements of the elevation of PSA over time. It was found that the PSA levels in serum increased more rapidly in men with cancer than in healthy men, which helped to differentiate between these two groups. A PSA velocity of 0.75 ng/ml per year or greater was strongly suggestive of cancer. However, to obtain reliable results, it was also found necessary to calculate the slope of the PSA elevation in serum, that is, the PSA velocity, from a minimum of three different measurements performed during a time period of at least 2 years. Still, there are several limitations of the method that restrict its clinical utility that include normal biological intraindividual day-to-day variation as well as interassay variability. Nixon *et al.*<sup>76</sup> showed in a study of 24 men that if an increase in PSA was less than 20 to 46%, it may be due to biological and analytical variation.

Based on the knowledge that PSA concentrations also increase with prostate volume and that more PSA is released per gland volume in patients with PCa than in those with BPH, Benson *et al.*<sup>77,78</sup> inaugurated a concept called PSA density in which the PSA concentration is divided by the trans-rectal ultrasound (TRUS)-measured volume of the prostate gland. Benson and co-workers found that a higher PSA density is indeed associated with a greater likelihood of PCa. However, analyzing PSA density is of limited value due to the following: it is difficult to make accurate prostate volume measurements by TRUS; the epithelium-to-stroma ratio varies in BPH; and only the epithelium produces PSA.<sup>79,80</sup> As this procedure also requires the use of ultrasound, it is less practical in the initial screening for PCa.

Transition zone PSA density is another PSA-derived method that has been proposed to facilitate discrimination of men with BPH from those with PCa.<sup>81,82</sup> In this analysis, PSA is divided by the volume of the transition zone of the prostate gland, because BPH is localized almost exclusively in that area of the gland. Poor reproducibility due to difficulties in providing accurate TRUS-based volume determinations of the transition zone also limits the usefulness of this type of density measurement.

### **III. FREE PSA**

#### **A. Free PSA for Early Detection of Prostate Cancer**

##### **1. Free PSA to Distinguish BPH from PCa**

Christensson *et al.* were the first to report on different molecular PSA forms in blood in 1990, where they demonstrated the ability of PSA to form covalent complexes with several major inhibitors such as AMG and ACT.<sup>35</sup> In 1991, Stenman *et al.* were the first to report that the proportion of complexed PSA (PSA-ACT) was higher in men with PCa than in those with BPH.<sup>42</sup> Independently, Lilja *et al.* were the first to report generation of monoclonal antibodies specific for free PSA, which subsequently were used to develop three different assays: one specific for detection of free PSA, one for PSA-ACT complexes, and one for total PSA.<sup>43</sup> Their data showed that the PSA-ACT complex is the predominant form of immunoreactive PSA in serum, accounting for a mean of about 85% of total PSA, while the free, noncomplexed PSA-form(s) accounted for the remaining approximately 15%.<sup>43</sup> Christensson *et al.* reported that the proportion of complexed PSA (PSA-ACT) was significantly higher in men with PCa than in those with BPH.<sup>83</sup> In agreement with this finding they also reported that the ratio of free-to-total PSA was statistically

significantly lower in 109 patients with PCa (a ratio of 0.18) than in 135 men with BPH (a ratio of 0.28)<sup>83</sup>; even when they excluded 43 patients from the heterogeneous group of PCa patients who received hormonal treatment the statistical significance was similar with a free-to-total PSA ratio of 16% in prostate cancer patients vs. 28% in men with benign conditions. The benign pathology was proven by transurethral resection of the prostate; however, not all had fine needle biopsies of the peripheral zone to safely rule out PCa at this location.<sup>83</sup>

Why there is a higher proportion of serum PSA complexed to ACT in patients with carcinoma of the prostate than in those with BPH still remains unclear. One possible explanation for this phenomenon could be that local contribution of ACT is more pronounced in prostate cancer cells than epithelial cells in BPH tissue.<sup>84,85</sup> However, in a recent investigation on tissue Stephan *et al.*<sup>86</sup> were unable to demonstrate the presence of any PSA-ACT complexes intracellularly in the cancer lesions and concluded that the complex formation presumably takes place after extracellular release of enzymatically active free PSA. Another possible explanation could be that BPH and prostate cancer cells secrete different forms of free PSA (enzymatically active or inactive forms), or at least different proportions of these. This theory is supported by the finding of different molecular forms of free PSA in blood.<sup>55,56</sup> It had been demonstrated previously that cell barriers are better preserved in benign conditions than in malignant lesions,<sup>87</sup> something that might also influence the different molecular forms detected in blood.

Many studies<sup>88-91</sup> that were conducted subsequently confirm the early data of Stenman *et al.*<sup>42</sup> and Christensson *et al.*<sup>83</sup> that the use of the free-to-total PSA ratio enhances the specificity of conventional total PSA testing, which may be used to avoid many unnecessary prostate biopsies. Some of these studies also demonstrated that the diagnostic enhancement contributed by the free-to-total PSA ratio was most pronounced at total PSA levels <10.0 ng/ml.<sup>88,92</sup> Conflicting results have been published on the benefits of free-to-total PSA ratio measurements at total PSA levels below 4.0 ng/ml.<sup>93-98</sup> Different free PSA/total PSA assays have different “cut-offs” — one variable in comparative studies. Another variable is different patient selection criteria. Also, in some cases the detection limit of the free PSA measurements may not be sufficiently low to allow generation of reliable data at low total PSA levels. Interestingly, Fowler *et al.* investigated racial differences of free PSA in a study including 222 African-Americans and 298 Caucasian-Americans all suspected of PCa with total PSA levels from 2.5 to 9.9 ng/ml, and showed that black men diagnosed with PCa by prostate biopsies had higher free-to-total PSA ratios than the white men<sup>99</sup>; furthermore, by applying a commonly used cut-off of 25% free-to-total PSA more early-stage PCa in black men would have been missed. In

a multicenter study, Catalona *et al.* investigated the performance of the free-to-total PSA ratio at total PSA levels from 4.0 to 10.0 ng/ml<sup>100</sup>, and reported that at a free-to-total PSA ratio of <25%, the sensitivity was 95%, while the specificity was enhanced by 20%. It was concluded from this study that at an expense of missing 5% of the cancer cases, 20% of the negative biopsies (i.e., false positives) could be avoided. Many subsequent studies focused on the use of free-to-total PSA for screening purposes.

## **2. Free PSA in Screening for Prostate Cancer**

Despite international efforts, early detection and treatment of PCa and strategies used in screening policies remain controversial. Adding new parameters to established screening parameters must help to decrease the proportion of men undergoing prostate biopsies and still allow detection of the majority of cancers, particularly those that are organ confined and aggressive, and therefore might be appropriate targets for curative therapy. Screening for PCa is usually performed by the conventional total PSA testing in blood and digital rectal examination (DRE) of the prostate. TRUS can be added for detection of lesions and volumetry of the prostate, but it has been demonstrated not to be cost-efficient as a primary screening tool. Measurements of the ratio of free-to total-PSA represent a novel parameter with potential to increase the specificity of the conventional total PSA testing in screening populations. Bangma *et al.* showed in 1726 men screened for PCa with PSA serum levels above 4.0 ng/ml that 39% of biopsies could have been omitted by missing 11% of cancers by using a cut-off of <20% for free-to-total PSA.<sup>101</sup> Catalona *et al.* applied a cut-off of <10% for free-to-total PSA to 193 men with PCa detected during screening on routine biopsies and normal digital examination<sup>100</sup>; the PPV was 46% meaning 46 nonpalpable cancers per 100 biopsies could be detected. Applying this strategy to the large screening population of 10,523 men in the European Randomized Study of Screening for Prostate Cancer, Schröder *et al.* obtained a dramatic effect in reducing the rate of unnecessary biopsies.<sup>102</sup> In a prospective screening study on 158 men, of which 106 had PSA levels between 2.5 and 10.0 ng/ml, 37 cancers were detected by prostate biopsies.<sup>103</sup> By using a cut-off of <18% for the free-to-total PSA ratio, a significant amount (25%) of cancers would have been missed; however, with a cut-off of 22% only 2% of cancers would have been missed, with an elimination of 30% of unnecessary biopsies. Looking at a screening population with total PSA levels <3.0 ng/ml, Törnblom *et al.* found no cancers over a cut-off of 18% free PSA ratio;<sup>98</sup> however, below this cut-off nine cancers could be found that comprised 14% of all diagnosed cancers.

In a literature review on the use of free PSA, Stein *et al.* very recently found not only a cost reduction of 30% during PCa screening in the U.S., but also that up to 48% of men would have been spared unnecessary biopsies with only very little reduction in cancer detection rate.<sup>104</sup> Some authors focused on the utility of percent free-to-total PSA measurements to avoid repeat biopsies for men with previously negative prostate biopsies.<sup>105–107</sup> Morgan *et al.* investigated 64 men with elevated PSA levels from 4.1 to 24.8 ng/ml, normal DRE and two or more previous negative sets of systematic sextant biopsies<sup>105</sup>; at a cut-off of <10% free-to-total PSA they reported a sensitivity of 91% and specificity of 86%. Using receiver operator characteristics (ROC) to calculate areas under the curves (AUCs), percent free-to-total PSA performed the best (AUC = 0.93) followed by PSA density (AUC 0.69). Catalona *et al.* looked at 163 men with total PSA levels from 4.1 to 10.0 ng/ml undergoing repeat biopsies<sup>106</sup>; 90% or 95% of the 20 cancer cases would have been detected if the percent free-to-total PSA cut-off would have been set at <10% or <8%, avoiding 12% or 13% of the unnecessary biopsies, respectively. The very recent prospective study by Djavan *et al.* included 820 men with total PSA levels from 4.0 to 10.0 ng/ml who underwent repeat biopsies<sup>107</sup>; 83 cancers were detected. At a cut-off of <30% percent free-to-total PSA, 90% of the cancers would have been detected and at the same time 50% of the unnecessary prostate biopsies would have been avoided. Here, the ROC analysis showed that the AUC for percent free-to-total PSA (AUC 0.75) was greater than those for transition zone PSA density (AUC 0.69), PSA density (AUC 0.62), or total PSA (AUC 0.60).

### **3. Free PSA for Staging and Grading of Prostate Cancer**

The ratio of free-to-total PSA has proven useful for enhancing the specificity of PSA to distinguish between malignant and benign prostatic diseases. Therefore, it was logical to evaluate the utility of this ratio for preoperative staging of PCa or even to estimate cancer volume. However, several investigators have come to slightly differing conclusions about the value of percent free-to-total PSA in predicting the final pathological stage using retrospective data of radical prostatectomy specimens<sup>87,108–112</sup> or using data from a prospective multicenter study.<sup>113</sup> Using pooled serum of patients with PCa at various stages, Stamey *et al.* showed that the fraction of serum PSA complexed to ACT remained relatively constant even when hormonal or radiation treatment had failed.<sup>108</sup> Lerner *et al.*<sup>109</sup> and Bangma *et al.*<sup>111</sup> found no statistically significant differences among pathological tumor stages and grades; however, Lerner *et al.* described statistically significant differences among pathological

substages.<sup>109</sup> Pannek *et al.* found that 97% of 301 patients had free-to-total PSA ratios <25%, which was consistent with the fact that they were considered suspicious for prostate cancer<sup>110</sup>; however, percent free-to-total PSA did not enhance the preoperative prediction of either organ-confined cancer or lymph node-positive disease compared with the conventional total PSA levels in serum. Data from the Department of Urology at the University Hospital in Hamburg, Germany, on 170 patients with clinically localized PCa showed no difference in the free-to-total ratio between pT2 and pT3 cancers<sup>112</sup>; however, there was a significantly lower ratio in pT3 cancers with seminal vesicle invasion compared with those without seminal vesicle invasion ( $p = 0.015$ ). Looking at cancer grade, there was a trend toward lower free-to-total PSA ratios with higher-grade cancers.<sup>112</sup>

In a preliminary study on 33 patients, Arcangeli *et al.* found significantly ( $p = 0.05$ ) lower free-to-total PSA ratios for cancer with capsular penetration than for those that were organ confined<sup>114</sup>; using a cut-off ratio of <14%, cancer with free-to-total PSA ratios below this cut-off had higher Gleason grades. Sothwick *et al.*<sup>113</sup> reported in their study of 268 patients with serum PSA 4.0 to 10.0 ng/ml and selected for radical prostatectomy, that higher percent-free PSA levels were associated with more favorable histopathological findings in prostatectomy specimens. A value of 15% free PSA provided the greatest discrimination in predicting favorable pathological outcome. Moreover, multivariate logistic regression revealed percent-free PSA to be the strongest predictor of postoperative pathological outcome (odds ratio 2.25), followed by biopsy Gleason sum (2.06) and patient age (1.35). Very recently, Grossklaus *et al.* found a significant inverse relationship between percent free-to-total PSA and tumor volume in a subset of 37 patients with total PSA levels >4.0 ng/ml.<sup>115</sup> Epstein *et al.* showed that the presence of insignificant cancer of the prostate, that is, defined as organ-confined cancer with tumor volume of <0.5 ml and no Gleason grade 4 or 5, could be accurately predicted preoperatively using a cut-off of >15% free-to-total PSA in conjunction with needle biopsy findings.<sup>116</sup>

#### **4. Influence of Prostate Volume, Age, and Inflammation of the Prostate on Free PSA**

It was reported by many investigators that age, and prostate volume may independently influence free-to-total PSA ratios.<sup>115,117-119</sup> Partin *et al.* showed an increase of percent free-to-total PSA with increasing benign gland volume of the prostate<sup>118</sup>; dividing the patients into two groups, they reached 95% sensitivity using a lower cut-off of >14% free-to-total PSA for small glands

(<35 ml prostate volume) and 25% free-to-total PSA for larger glands ( $\geq 35$  ml of prostate volume). Similar findings were reported by Haese *et al.* on 395 patients who all had histology proven disease by six systematic biopsies (n = 156 BPH, n = 239 PCa)<sup>117</sup>; however, they used stepwise analysis for each 10 ml of prostate volume. The lower percent free-to-total PSA in PCa compared with BPH patients was found to be statistically significant only for prostate gland volumes up to 60 ml; there were no statistically significant differences in percent free-to-total PSA in PCa compared with BPH patients for the larger glands (gland volume >60 ml). Consistent with these findings, ROC curves for percent free-to-total PSA in these two groups showed larger AUCs for percent free-to-total PSA for the patients with small volume prostates compared with larger ones. Ornstein *et al.* similarly found increasing percent free-to-total PSA with increasing prostate volume (<50 ml vs.  $\geq 50$  ml of prostate volume) in 67 BPH patients (p = 0.008).<sup>119</sup>

Partin *et al.* also reported an increasing percent free-to-total PSA with increasing patient age<sup>118</sup>; using a cut point of <18% free-to-total PSA, the sensitivity decreased (90% sensitivity), and many cancers were missed in patients over 67 years. This phenomenon could largely be related to increasing prostate volume with age; however, in the study by Partin *et al.* the correlation between volume and age was insignificant for men with total PSA levels from 4.0 to 10.0 ng/ml.<sup>118</sup> Vashi *et al.* reported similar data based on a study of 225 men with biopsy-proven BPH<sup>120</sup>; percent free-to-total PSA increased with increasing age at total PSA levels from 2.0 to 20.0 ng/ml. However, no correction was made for prostate gland volume as a putative contributing factor. Oesterling *et al.* and Lein *et al.* reported some contradictory data for healthy men<sup>121,122</sup>; they found increasing total and free PSA levels for men from 20 to 89 years of age, but found no significantly different percent free-to-total PSA from the third to eighth decades.

Total PSA levels have been reported to be elevated in acute prostatitis.<sup>123</sup> Some authors have also investigated the influence on percent free-to-total PSA by acute inflammation of the prostate. Ornstein *et al.* similarly found that in men with BPH, percent free-to-total PSA was significantly higher in men with a prostate gland larger than 50 ml (n = 35) than in men whose gland was smaller than 50 ml (n = 28) (p = 0.008)<sup>119</sup>; however, all patients with histological signs of acute inflammation had moderately elevated PSA levels in serum (mean of 5.6 ng/ml; SD  $\pm$  1.6 ng/ml) but no clinical signs of acute inflammation. The percent free-to-total PSA (22%) was as high as for those with chronic inflammation (22.3%). In contrast, data reported by Fink *et al.* who studied 28 patients with acute and chronic inflammation of the prostate<sup>124</sup>; showed a mean percent free-to-total PSA of 51.3% that was normalized within 2 weeks in parallel with relief from clinical symptoms of the disease.

## **5. Free PSA and Recurrence of Prostate Cancer**

Few data have been reported on free PSA as a marker for recurrence after treatment of prostate cancer with radical prostatectomy. Men with recurrent PCa should harbor “pure” cancer in that they have no benign prostate tissue. Alternatively, if the increase in free PSA in benign prostatic disease is due to compartmentalization of PSA in the transition zone (BPSA) the removal of the prostate changes the paradigm. “Clones” of metastatic cancer cells will now determine the amount of inactive (free) PSA released into the serum.

Preliminary data from the Hamburg University Department of Urology on 20 patients after pT3 pN0 radical prostatectomy showed a median 21% free-to-total PSA, ranging from 3.3 to 46.6%.<sup>125</sup> Interestingly, Vashi *et al.* demonstrated in 46 men with recurrent disease a mean of 9.7% in free-to-total PSA, and that 65% of the patients with recurrent PCa had <10% free-to-total PSA. However, four patients (9%) with more aggressive cancer and seminal invasion had >20% free-to-total PSA.<sup>126</sup> Also, Lin *et al.*<sup>127</sup> studied the different forms of PSA occurring in postoperative sera from 52 men with elevated PSA concentrations after radical prostatectomy. 52% of the patients had percent free <15%, 48% had percent free >15 and 13% had levels >30%. No significant relationship was found between percent free PSA and grade, stage, or severity of disease; however, percent free PSA was significantly increased in patients receiving hormonal treatment and/or radiation therapy versus those who received no treatment.

## **6. When to Use Free PSA?**

A distinct relationship has been established linking percent-free PSA to the probability of PCa; patients with low percent free-to-total PSA have far higher probability of having PCa than those with higher ratios. However, some factors such as age, prostate volume, and acute inflammation may influence percent free-to-total PSA that must be taken into account by the interpreting physician. The data reviewed consistently show that percent free PSA enhances the specificity of total PSA testing using established cut-off ratios that result in reduced need for prostate biopsies, thus subsequently reducing costs. However, percent-free PSA is not an ideal marker for staging or grading of PCa preoperatively or helpful when estimating recurrent disease.

### **B. Measurements of PSA-ACT Complexes (Complexed PSA)**

Several problems remain despite the advance of free-to-total PSA. High analytical precision of free PSA assays is important, in particular in low ranges

of free PSA levels, to reduce the overlap in percent free-to-total PSA between men with and those without PCa. Further, intraindividual day-to-day variation is also likely to be higher for free PSA compared with PSA-ACT due to the short half-life of 12 to 18 h in plasma for free PSA compared with very slow elimination of PSA-ACT complexes.<sup>62</sup> Moreover, limited *in vitro* stability of free PSA in serum<sup>128,129</sup> compared with the significantly higher stability of PSA-ACT requires compliance with established handling of clinical serum specimens to avoid falsely decreased free-to-total ratios, or taking advantage of the enhanced stability of free PSA in anticoagulated EDTA or heparin plasma samples. The free PSA level is significantly decreased following 24 h storage unless serum is stored frozen, while there is no decrease in PSA-ACT levels up to 7 days storage.<sup>128,129</sup> However, 2 years of storage at  $-70^{\circ}\text{C}$  has been shown to give acceptable levels of both free and total PSA.<sup>130</sup> Further, problems with lack of uniformity of manufactured free PSA assays is amplified when free PSA levels are combined with total PSA levels to obtain free-to-total ratios, which becomes more problematic when total PSA assays do not detect free and complexed PSA forms with equimolar signal intensity.

The data first reported by Stenman *et al.*, Christenson *et al.*, and Leinonen *et al.*<sup>42,83,131</sup> on the specific PSA-ACT complex measurements showed it to contribute significant diagnostic enhancement over the conventional total PSA testing. However, there was an overreading of PSA-ACT complex levels in these original reports that in subsequent reports was demonstrated to result from difficulties in eliminating nonspecific (i.e., false positive) background signals contributed by granulocyte-derived proteases, for example, cathepsin G, which also form covalent linkages to ACT and attach to the solid phase surfaces of micro-titer wells. However, Allard *et al.*<sup>132</sup> from Bayer Diagnostics reported on the design of an indirect immunodetection of complexed PSA (cPSA) in serum: an assay that does not recognize free PSA in the sample, but recognizes complexed PSA, in particular PSA-ACT. However, it has not yet been fully clarified whether this assay recognizes PSA-ACT and PSA-API complexes in an equimolar fashion. However, the PSA-ACT complexes are by far predominant as the PSA-API complexes contribute only 1 to 2% of total PSA.<sup>48,133</sup> The cPSA-assay employs a free-specific monoclonal antibody to enable selective shielding of free PSA from binding to the capture antibody linked to magnetic particles. Therefore, only complexed PSA remains to bind to the polyclonal indicator antibody in the cPSA assay.

Assay validation<sup>134</sup> ( $n = 300$ ) has shown that the sum of the Bayer cPSA concentration plus the Beckman Coulter free PSA concentration equals that of the Beckman Coulter total PSA concentration ( $y = 1.05x - 0.25$ ;  $r = 0.99$  in the 0 to 25 ng/ml PSA range). Complex PSA measurements offer advantages in regard to stability as PSA-ACT is far more stable in the test tube than free

PSA.<sup>128,129</sup> Also, cPSA levels are much less affected by prostatic manipulation such as DRE, cystoscopy, or biopsy compared with a significant increase in total PSA subsequent to these procedures.<sup>135</sup> The evaluation of the cPSA assay using ROC showed consistently greater AUCs (although not always statistically significant greater) than AUCs of total PSA in each of ten different studies evaluating samples from close to 2000 men.<sup>134,136–144</sup> However, cPSA was not as specific or sensitive as percent-free PSA or percent-complexed PSA in the total PSA “gray zone” (e.g., 4 to 10 ng/ml). One of these studies incorporated 272 men with biopsy-detected carcinoma and 385 men with benign findings.<sup>138</sup> The enhancement in test performance contributed by the cPSA assay in the truncated 4 to 10 ng/ml range of PSA levels shows enhanced capacity of cPSA to discriminate men with cancer from men without cancer compared with conventional total PSA measurements.<sup>138</sup> Another of these studies included 367 men with biopsy-detected carcinoma and 290 men with BPH (enlarged prostates but benign biopsy findings).<sup>136</sup> Here, in the truncated range of 2 to 20 ng/ml or in the truncated range of 4 to 10 ng/ml, only percent-free PSA had an AUC significantly greater than total PSA. Percent cPSA or cPSA alone gave larger AUCs than that for total PSA, but no statistical significance was seen. Conflicting results on the utility of cPSA in these two large studies may be due to different patient selection criteria or different methods for the determination of the PSA forms.

#### **IV. HUMAN GLANDULAR KALLIKREIN 2 (hK2)**

In 1987, Schedlich and associates<sup>1</sup> identified the gene coding for hK2, or hGK 1, as it was called at that time. Soon thereafter it was found that hK2, like PSA, is expressed mainly in prostatic tissue,<sup>7,145</sup> and hK2 is expressed in the prostate tissue at levels corresponding to 10 to 50% of the expression of PSA.<sup>7–9</sup> However, it was not until 1995 that the protein was isolated from seminal plasma,<sup>146</sup> in which it was found to occur mainly in complex with protein C inhibitor (PCI). In 1990, Henttu *et al.*<sup>8</sup> reported that there was good correlation between expression of PSA and hK2 mRNA in BPH tissue, but that correlation was not as marked in PCa tissue. In 1997, Darson *et al.*<sup>147</sup> further examined the expression of hK2 and PSA in prostate tissue and found that, in contrast to PSA, hK2 is expressed at higher levels in prostatic adenocarcinoma than in the normal prostate epithelium. Therefore, the data suggest that the hK2 expression is incrementally increased during the transition from benign epithelium to primary cancer and lymph node metastases.<sup>147,148</sup> Like PSA, hK2 is found in seminal plasma,<sup>146</sup> although at much lower concentrations (i.e., the

hK2 levels are about 1% of the PSA concentration),<sup>149</sup> and several studies have also shown that hK2 levels in serum are elevated in men with prostatic disease.<sup>96,150–160</sup>

## A. Biochemical and Physiological Characteristics

The hK2 mRNA codes for a 261 amino acid prepro form of hK2, whereas the mature protein is composed of 237 amino acids and has a single glycosylation site at Asn<sub>78</sub>.<sup>1</sup> Mature hK2 has an apparent molecular mass of 31 to 33 kDa according to SDS-PAGE<sup>9,161,162</sup> and a true molecular mass of 28.5 kDa determined by laser desorption mass spectrometry.<sup>163</sup> The protein is initially synthesized with a signal peptide, which is removed after transfer to the ER, and a propeptide that is removed after activation. Activation of hK2 is believed to be an autocatalytic process,<sup>23,164,165</sup> but it is not known where it occurs.

The hK2 molecule has an aspartate residue at the bottom of its catalytic pocket, which is critical for the restricted trypsin-like substrate specificity of classic kallikreins.<sup>1</sup> The enzymatic activity of hK2 catalyzes cleavage of substrates on the C-terminal side of certain single and double arginines, and, much less often, lysines and histidines.<sup>166</sup> Recent investigations have revealed many potential biological functions of hK2. Studies *in vitro* have shown that hK2 can convert the zymogen form of PSA into an enzymatically active protein.<sup>22–24</sup> The significance of this finding *in vivo* is not yet known, although hK2 is likely one physiological activator of PSA, considering the co-localization of these two proteins in the prostate, although other proPSA converting pathways may also exist. Recently, it was shown that prostin, a novel human prostatic serine protease, also can activate proPSA.<sup>25</sup> Similar to PSA, hK2 can cleave the semenogelins and fibronectin,<sup>166,167</sup> but the cleavage pattern and identified cleavage sites in semenogelin I and II generated by the action of hK2 *in vitro* are distinct from those generated by PSA. Further, the few cleavage sites identified thus far in the semenogelin in liquefied seminal plasma are generated by the action of PSA and not by hK2.<sup>30</sup> Another potential function of hK2 is regulation of the urokinase plasminogen activator (uPA) system. It has been reported that hK2 activates uPA and inactivates PAI-1, the primary inhibitor of uPA.<sup>24,168,169</sup> It has also been shown that hK2 exhibits kininogenase activity<sup>170</sup> by releasing bradykinin from high-molecular-weight kininogen (HMWK). One of the many effects of bradykinin is to increase sperm motility,<sup>171</sup> which is why it is possible that hK2 has a physiological function in seminal fluid.

Deperthes<sup>146</sup> found that the hK2 in seminal plasma was inactive and that most of hK2 is complexed with PCI. Later, *in vitro* studies showed that hK2

can form complexes with several extracellular protease inhibitors besides PCI, including  $\alpha_2$ -antiplasmin ( $\alpha_2$ -AP), AMG, ACT, anti-thrombin III (ATIII), C1-inactivator and PAI-1.<sup>163,168,169,172,173</sup> Complex formation between hK2 and  $\alpha_1$ -antitrypsin (API) has not been reported. The most rapid inhibition occurs when hK2 is complexed with PCI, PAI-1, or AMG.<sup>169,172</sup> The enzymatic activity of hK2 can also be regulated by micromolar levels of  $Zn^{2+}$ , which is noteworthy because zinc is present at millimolar levels in the prostate and in seminal fluid.<sup>166</sup> Moreover, in a recent investigation,<sup>174</sup> approximately 10% of the hK2 in prostate tumor tissue was found in complex with the intracellular serine protease inhibitor-6 (PI-6) and may reflect tumor necrosis.

## B. Characteristics of hK2 in Blood

Most of the immunodetectable hK2 in serum appears to be in a free, noncomplexed 30-kDa form. It has been proposed that catalytically inactive prohK2 might represent a significant portion of free hK2 in serum, both in men with BPH and those with PCa.<sup>153</sup> The limited data reported suggest that only a small fraction of the hK2 (about 5 to 20%) has a 90-kDa size and is found in complex with proteinase inhibitors, possibly as hK2-ACT complexes.<sup>150,173,175,176</sup> Therefore, the importance of extracellular protease inhibitors for the regulation of hK2 in serum is not yet understood.

As most of hK2 detected in serum appears to be in the free form,<sup>150,175,176</sup> it is likely that elimination of hK2 from blood could mainly occur by glomerular filtration, although only very little data have been reported on the clearance of hK2 from serum. In a study by Lilja *et al.*,<sup>52</sup> the prostatic manipulation during prostatectomy caused an almost tenfold increase in levels of free PSA, but it did not elevate hK2 concentrations; in fact, within 2 h of surgery, the mean hK2 levels were below the functional detection limit of the assay (0.05 ng/ml). In that investigation, the very low concentrations of hK2 in serum, in combination with the limited functional sensitivity of the available assay, made it impossible to calculate the elimination rate, although hK2 did seem to be rapidly cleared from blood circulation.

## C. Clinical Applications

The striking similarities between hK2 and PSA, along with the observed differences in expression of these two proteins in the prostate epithelium,<sup>147,148</sup> have resulted in much speculation on the potential role of hK2 as a novel marker for PCa. Immunodetection of hK2 was initially difficult due to the

extensive structural similarity of the two proteins, about 80% identity in primary structure, which might result in severe immunological cross-reaction with PSA. Production of recombinant hK2<sup>161,177-179</sup> made it possible to characterize the antigenic epitope structures of the protein in greater detail and to evaluate the cross-reactivity of monoclonal anti-PSA antibodies with hK2.<sup>161,179-181</sup> The information generated on the binding specificity of different monoclonal antibodies has allowed the design and development of highly sensitive and specific methods for the detection of each of the two proteins in tissues and in different body fluids.<sup>147,148,150,152,156,173,175,176</sup> The concentration of hK2 in serum is much lower than the level of PSA (i.e., about 1 to 2%).<sup>150,154,158</sup> However, the covariance of hK2 and PSA levels is usually much less than 60%,<sup>150,154-156,159,176</sup> which indicates that hK2 might contribute independent information, perhaps in an additive fashion, to further enhance the detection of prostate cancer.

Many studies have demonstrated that hK2-levels in serum are elevated in men with prostate disease.<sup>96,150-160</sup> Also, it has been shown that concentrations of hK2, like PSA, increase with age<sup>156,175</sup> and are very low in female sera, as well as in sera of healthy<sup>152,156,175,176</sup> and prostatectomized<sup>156,175</sup> men. Furthermore, PCa patients have higher hK2 levels than men with BPH.<sup>152,154,155,158</sup> In 1998, Kwiatkowski *et al.*<sup>154</sup> showed that combining the hK2 levels with free PSA levels was superior to measurements of percent free PSA for the discrimination of symptomatic men with PCa from those without cancer among men who had PSA levels from 4 to 10 ng/ml. Later, it was demonstrated<sup>158</sup> that discrimination of men with BPH from those with localized PCa was significantly improved by combining the measured levels of hK2 with those of both free and total PSA.<sup>96,159</sup> Two recent studies indicate that hK2 determinations may be helpful in predicting organ-confined disease in the preoperative staging of PCa patients. Haese *et al.*<sup>182</sup> were the first to report that hK2 is useful for distinguishing between organ-confined and non-organ-confined tumors. This finding was confirmed in a study by Recker *et al.*<sup>183</sup> that also showed that the hK2 levels in addition to free and total PSA-levels were useful to improve the discrimination of grade-G1 from grade-G3 tumors and of grade-G2 from grade-G3 tumors.

#### **D. Extraprostatic Sources of Prostate-Specific Antigen (PSA) and Human Glandular Kallikrein 2 (hK2)**

The name “prostate-specific antigen” reflects the initial widespread belief that the expression of this protein was restricted to the prostate gland. It has now been well documented that PSA is not prostate specific; it is

expressed in many tissues, although at lower levels, and is secreted in many biological fluids besides seminal plasma. A list of tissues expressing PSA and hK2 is presented in Table 1.<sup>69,175,184–220</sup> A list of biological fluids (serum values from normal subjects) containing PSA and hK2 is shown in Table 2.<sup>156,175,186,187,219–230</sup>

The periurethral (Skene's) gland was the first female tissue that was reported to produce PSA.<sup>199</sup> This tissue has been referred to as 'the female prostate'<sup>200</sup> as its developmental origin is common to that of the male prostate. PSA is detectable in healthy breast tissue and is present in breast tumor extracts.<sup>229</sup> Various breast secretions contain PSA, including nipple aspirate fluid, milk of lactating women, and breast cyst fluid. Endometrial tissue produces PSA as do some ovarian tumors, and PSA is present in amniotic fluid. Low levels of circulating PSA are detectable in female sera and in some cerebrospinal fluids (for specific references see Tables 1 and 2).

Numerous studies have already indicated that the phenomenon of PSA production by breast tissue can be reproduced with breast carcinoma cell lines.<sup>231–233</sup> It is already known that the PSA gene is regulated by androgens<sup>234</sup>; with breast carcinoma cell lines, it has been shown that PSA is up-regulated

**TABLE 1**  
**Tissues Expressing PSA and hK2**

<b>Tissue</b>	<b>PSA reference</b>	<b>hK2 reference</b>
Prostate	69, 184–185	220
Breast	186–189	175
Thyroid	190	190
Lung	191–194	—
Ovary	195–197	—
Urethra/periurethral/paraurethral glands	198–205	—
Apocrine sweat glands	206	—
Urachus	207	—
Bladder	208–210	—
Cloacogenic glandular epithelium/ anal gland	211	—
Salivary gland	212–214	—
Male accessory sex glands	215	—
Uterus/endometrium	210, 216	—
Adrenal/colon/kidney/liver/parotid	191	—
Bone marrow	217	—
Pancreas	210, 218–219	—
Bile duct	210	—

**TABLE 2**  
**Biological fluids containing PSA and hK2<sup>a</sup>**

Fluid	PSA Levels (mg/l)			hK2 Levels (mg/l)		
	Range	Median	Ref.	Range	Median	Ref.
Seminal plasma	500,000–3,000,000	800,000	219, 220	2,000–12,000	6,000	175, 220
Nipple aspirate fluid	0–13,000	500	221	0–171	20	230
Breast cyst fluid	0–42	0.08	222	0–21	0.10	222
Urine — males	—	20	223	—	1.0	223
Urine - females	0–0.10	0.017	224	<0.006	<0.006	224
Breast milk	0–350	0.47	225	0–2.7	0.021	222
Amniotic fluid	0–16	3 <sup>b</sup>	226	0–0.38	0	222
Cerebrospinal fluid	0–0.4	0	227	—	—	—
Serum — males	0–4	0.70	228	0–0.24	0.026	156
Serum — females	0–0.010	0.001	224	<0.006	<0.006	224
Breast tumor cytosols	0–12	0.005	186, 187, 229	0–7	0.007	230

<sup>a</sup> We refer to healthy populations in most cases. See references for more details.

<sup>b</sup> At gestational week 18.

by androgens and progestins and, to a lower extent, by mineralocorticoids and glucocorticoids.<sup>231–234</sup>

The physiological role of PSA in extraprostatic tissues is not known. A recent review summarizes current knowledge, which, at times, seems conflicting.<sup>235</sup> It is clear that PSA has biological functions beyond semen liquefaction, which is the accepted function of this enzyme in seminal plasma.<sup>33</sup>

PSA levels in various fluids, and especially in female serum, are extremely low and they are difficult to measure with conventional PSA assays. However, with the advent of highly sensitive immunological assays for PSA, it has now been documented that PSA is present in female serum.<sup>236–240</sup> In some cases, these levels approach those of males.<sup>241</sup> These ultrasensitive PSA assays have facilitated studies toward investigating the diagnostic and prognostic value of PSA in females.

## **V. PSA AND THE BREAST**

### **A. PSA as a Prognostic Indicator in Breast Cancer**

PSA immunoreactivity in breast cancer cytosolic extracts was first identified in 1994.<sup>186,187</sup> More recent studies with new, ultrasensitive immunoassays demonstrated that approximately 70% of breast tumor cytosolic extracts contain immunoreactive PSA.<sup>242</sup> The localization of breast tumor PSA was accomplished using immunohistochemistry with anti-PSA antibodies.<sup>243–246</sup> The presence of PSA in breast tumors, as identified by immunoassays, correlated with that detected immunohistochemically.<sup>243</sup> Western blots and high-performance liquid chromatography demonstrated that breast tumor PSA was of the same molecular weight as seminal PSA (vast majority in the free-PSA form).<sup>187</sup> Molecular analysis verified that the mRNA of breast tumor PSA was identical in sequence to prostatic PSA.<sup>188</sup> DNA sequencing further confirmed that no mutations were present in the coding region of the PSA gene in breast tumors.<sup>247</sup>

PSA positivity in breast tumor cytosols is weakly associated with the presence of estrogen and progesterone receptors.<sup>186,248</sup> In addition to the *in vitro* models, which have established the hormonal regulation of PSA in breast carcinoma cell lines,<sup>231–233</sup> a severe combined immunodeficiency (SCID) mouse model has confirmed these data as well.<sup>249</sup> The association between steroid hormone receptors and PSA production was also demonstrated *in vivo* in breast tissue.<sup>189</sup> PSA is expressed at low levels by healthy mammary tissue.<sup>250</sup> Breast cytosolic extracts from women receiving progestin-containing oral contraceptives had considerably more PSA immunoreactivity, confirming the

hormonal dependence of PSA production.<sup>189</sup> The prognostic value of PSA measurement in breast cytosols was examined in three studies.<sup>242,251,252</sup> PSA positivity in primary breast tumors is significantly associated with smaller tumors, steroid hormone receptor positivity, low cellularity, diploid tumors, low S-phase fraction, less advanced disease stage, younger patient age, lower risk for relapse, and longer overall survival. Similarly, low levels were more often found in larger tumors, tumors of older, post-menopausal patients and in steroid hormone receptor-negative tumors. All these data suggest PSA to be a favorable prognostic indicator in breast cancer patients. Smaller, immunohistochemical studies found an association of PSA with androgen receptors,<sup>246</sup> association with unfavorable prognosis in a subset of patients with absence of PSA and estrogen receptors,<sup>245</sup> or of no prognostic value.<sup>244</sup> The differences are possibly due to [a] methodology and [b] size of the patient groups.

Based on these findings, another study examined the predictive value of PSA measurements in cytosolic extracts in relation to tamoxifen response.<sup>248</sup> High levels of PSA in primary breast tumors were related to poor response, short duration of response, and poor overall survival after tamoxifen therapy in recurrent disease. Given that previous studies have shown that PSA is a favorable marker of prognosis in breast cancer, the finding that PSA is elevated in tumors and disease that is refractory to tamoxifen was unexpected, with no obvious explanation at present.

Another two reports shed some more light into the prognostic value of PSA in breast cancer. In one study, serum PSA was examined as a prognostic indicator in women with metastatic breast cancer who were receiving the synthetic progestin megestrol acetate.<sup>253</sup> It was found that approximately 50% of these patients were able to increase their serum PSA severalfold, due to the administration of the drug. The patients who did respond with increased serum PSA had much worse prognosis and reduced survival than those who did not increase their serum PSA. In this study, serum PSA was taken as a conveniently measurable surrogate marker of tumor tissue PSA. This investigation had indicated that the effectiveness of the drug can be predicted by serum PSA measurements, but the result was also unexpected, because tumors that produced more PSA and secreted it into the circulation had a worse prognosis. This was investigated further in one patient who had a remarkable increase in serum and cerebrospinal fluid PSA after megestrol acetate administration.<sup>254</sup> This patient died very quickly from metastatic breast cancer. These data further indicate a possible role of PSA in breast cancer progression, but the mechanisms are still obscure.

## **B. PSA for Breast Cyst Classification**

Breast cyst fluid contains variable amounts of PSA.<sup>222,255–258</sup> It has also been indicated that of the two types of breast cysts, type I (secretory/apocrine) contain more PSA than type II (transudative/flattened).<sup>256,258–260</sup> Also, type I cysts have more free PSA than type II cysts.<sup>256</sup> It appears that the total amount of PSA as well as the percent of free PSA in breast cyst fluid has some value for cyst subclassification.

## **C. PSA in Nipple Aspirate Fluid and Breast Cancer Risk**

The majority (75%) of nipple aspirate fluids contain immunoreactive PSA.<sup>221,261,262</sup> Concentrations up to 13 mg/l have been reported, making nipple aspirate the biological fluid with the second highest concentration of PSA (after seminal plasma). Nipple aspirate fluid PSA levels are higher in premenopausal, in comparison to postmenopausal women, presumably due to higher levels of circulating steroid hormones before the menopause.<sup>221,261</sup> The concentration of PSA in nipple aspirate fluid appears to be inversely associated with breast cancer risk.<sup>221</sup> Nipple aspirate fluids from women with no risk factors for breast cancer had relatively high PSA concentrations, while women with breast cancer were more likely to have low PSA levels in nipple aspirate fluid. These findings suggest that PSA analysis in nipple aspirate fluid may constitute a potential tool for breast cancer risk assessment.

## **D. Serum PSA as a Marker of Breast Disease**

Using ultrasensitive PSA immunoassays, it has been demonstrated that at least 50% of normal female sera contain detectable PSA.<sup>224,236,238,239</sup> The likely source of circulating PSA in females is the mammary duct system, as PSA is expressed predominantly in breast tissue and enters its secretions. Serum PSA-levels in females are approximately 100 to 1000 times lower than male PSA levels in serum.

PSA is differentially expressed during the menstrual cycle, as the rise in serum PSA levels follows the progesterone concentration peaks.<sup>263</sup> PSA levels were found to increase in the sera of pregnant women,<sup>264</sup> and furthermore serum PSA is elevated in serum of both male and female newborns.<sup>265</sup>

Serum PSA levels are elevated in a fraction of endocrine-dependent diseases, including breast cancer, breast cystic disease, and uterine leiomyoma.<sup>239,266,267</sup> Furthermore, women with high levels of circulating andro-

gens consequently exhibiting hirsutism have augmented PSA levels in serum.<sup>268-270</sup> Lehrer *et al.* used reverse-transcription polymerase chain-reaction methodology to detect PSA transcripts in circulating cells of breast cancer patients and found that 25% of the cancer patients were positive.<sup>271</sup> This may aid in the molecular staging of breast cancer, similar to the situation with PCa.<sup>272</sup> Others have tried PSA mRNA as a marker of lymph node micrometastasis of breast cancer<sup>273</sup> and found a positive signal in approximately 60%.

Prostate-specific antigen circulates in blood in various forms, including PSA complexed to ACT and free PSA.<sup>266,267</sup> The measurement of the percent-free PSA in PCa patients is already used for the differential diagnosis between prostate cancer and benign prostatic hyperplasia.<sup>220</sup> The molecular forms of PSA have been investigated in serum of patients with breast cancer.<sup>239,266,267</sup> Using high-performance liquid chromatography and ultrasensitive immunoassay techniques, it was found that most breast cancer patients who have measurable PSA have free PSA as the predominant molecular form. This finding has been proposed as a way to diagnose breast cancer,<sup>267</sup> but the diagnostic sensitivity of this test is low, despite the fact that the diagnostic specificity is acceptable. The mechanism of this differential presence of PSA molecular forms in serum of breast cancer patients is not currently known.

## **VI. PSA IN AMNIOTIC FLUID**

Human amniotic fluid contains detectable amounts (median concentration approximately 3 ng/ml) of PSA.<sup>226,264,274</sup> The concentration of PSA increases with gestation from week 11 to 21, at which time it reaches a plateau, or slowly drops. The physiological background to this is not understood.

It was initially hypothesized that PSA measurements in maternal serum may be a useful screening parameter in order to identify mothers at risk of carrying fetuses affected with various abnormalities.<sup>275</sup> However, these findings have not been confirmed in subsequent studies despite the fact that amniotic fluid PSA was detected and found to be lower in patients affected with Downs syndrome.<sup>276,277</sup>

### **A. Extraprostatic Sources of Human Glandular Kallikrein 2 (hK2)**

hK2 was initially considered to be a prostatic-specific biomarker,<sup>220</sup> but it is now known that this protein is expressed extraprostatically as well. In

particular, hK2 has been detected in normal and malignant breast tissue extracts<sup>230</sup> and in breast milk, amniotic fluid, breast cyst fluid, serum, urine, and saliva.<sup>149,222</sup> These data suggest that diseased and normal breast secretions contain both PSA and hK2.

Breast carcinoma cell lines that produce hK2 have been identified,<sup>233</sup> and it has already been established that hK2 regulation is similar to that reported for PSA, namely, up-regulation by androgens, progestins, glucocorticoids, and mineralocorticoids. In all tissues and fluids in which hK2 has been detected, statistically significant correlation between PSA and hK2 values has been found.<sup>149,222</sup>

Studies have indicated that both PSA and hK2 are up-regulated after testosterone administration in female to male transsexuals (the increases are measurable in both serum and urine).<sup>224</sup> On the other hand, administration of antiandrogens and estrogens in male-to-female transsexuals causes dramatic reductions in the levels of PSA and hK2 in serum and urine.<sup>223</sup>

By using reverse-transcription polymerase chain-reaction technology, it has been reported that transcripts of PSA and hK2 can also be found in the thyroid.<sup>190</sup> These data clearly demonstrate that, although these two kallikreins show their highest expression in prostate tissue, numerous other tissues also produce these proteins. Therefore, it will be interesting to delineate the possible biological function of these kallikreins in nonprostatic tissues and other biological fluids.

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