DEVELOPMENT OF NOVEL ASSAYS FOR MEASURING DIFFERENT MOLECULAR FORMS OF PROSTATE SPECIFIC ANTIGEN

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Academic Dissertation

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1. LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals (I-IV)

I. **Zhu L**, Leinonen J, Zhang WM, Finne P, Stenman UH. Dual-label immunoassay for simultaneous measurement of prostate-specific antigen (PSA)-α1-antichymotrypsin complex together with free or total PSA. Clin Chem. 2003; 49(1): 97-103.

II. Wu P, **Zhu L**, Stenman UH, Leinonen J. Immunopeptidometric assay for enzymatically active prostate-specific antigen. Clin Chem. 2004; 50(1): 125-9.

III. **Zhu L**, Koistinen H, Wu P, Närvänen A, Schallmeiner E, Fredriksson S, Landegren U, Stenman UH. A sensitive proximity ligation assay for active PSA. Biol Chem. 2006; 387(6): 769-72.

IV. **Zhu L**, Koistinen H, Landegren U, Stenman UH. Proximity Ligation Measurement of the Complex between Prostate Specific Antigen and α1-Protease Inhibitor. Clin Chem. 2009; 55(9): 1665-1671.

2. ABBREVIATIONS

AUC	area under the curve
A2M	α2-macroglobulin
ACT	α1-chymotrypsin
ANN	artificial neural network
API	α1-protease inhibitor
BPH	benign prostatic hyperplasia
BPSA	benign PSA
CV	coefficient of variation
DRE	digital rectal examination
fPSA	free prostate specific antigen
hK2	human glandular kallikrein 2
IFMA	immunofluorometric assay
IPMA	immunopeptidometric assay
kD	kilodalton
KLK	kallikrein
MAb	monoclonal antibody
PCa	prostate cancer
proPSA	precursor PSA
PSA	prostate specific antigen
ROC	receiver operation curve
Serpin	serine protease inhibitor
TBS	Tris-buffered saline
tPSA	total prostate specific antigen
TRUS	transrectal ultrasound

3. SUMMARY

Measurement of prostate specific antigen (PSA) is a very sensitive method for diagnosing and monitoring of prostate cancer (PCa), but the specificity needs improvement. Measurements of different molecular forms of PSA have been shown to improve differentiation between PCa and benign prostatic diseases. However, accurate measurement of some isoforms has not been achieved in previous assays. The aim of the present study was to develop new assays that reliably measure enzymatically active PSA, PSA-α1-chymotrypsin (PSA-ACT) and PSA-α1-protease inhibitor (PSA-API), and to evaluate their diagnostic value.

We produced a novel monoclonal antibody (MAb), with better specificity for PSA-ACT and reduced reactivity with free ACT and cathepsin G-ACT. Double-label immunofluorometric assays using this MAb and another antibody to either free PSA (fPSA) or total PSA (tPSA) were developed and used to measure PSA-ACT and fPSA or tPSA at the same time. These assays provide enough sensitivity for measurement of PSA-ACT in sera with low PSA levels. The results obtained confirmed that proportion of PSA-ACT to tPSA (%PSA-ACT) was as useful as proportion of fPSA to tPSA (%fPSA) for discrimination between PCa and benign prostatic hyperplasia (BPH).

We developed an immunoassay for detection of PSA-API based on proximity ligation, which improved assay sensitivity 10-fold compared with conventional assays. A new anti API MAb together with an anti PSA MAb were used to detect PSA-API captured on the solid phase by another anti PSA MAb. Concentrations of PSA-API in serum samples with tPSA concentrations of 4-10 μg/L could be reliably measured. Our results confirmed previous findings that the PSA-API level is somewhat lower in men with than without PCa, and the combination of %fPSA and proportion of PSA-API to tPSA (%PSA-API) provides diagnostic improvement compared with either method alone. Assays based on this principle should be applicable to other immunoassays in which the nonspecific background is a problem.

Enzymatically active PSA has been shown to be a potential marker for PCa. A PSA-binding peptide fusion protein labeled with Eu^{3+} was used as a tracer to develop an immunopeptidometric sandwich assay for measurement of enzymatically active PSA. This assay showed high specificity, but sensitivity was not good enough for measurement of PSA concentrations in the gray zone, 2-10 μg/L, in which tPSA does not efficiently differentiate between PCa and BPH. To improve sensitivity, we further developed a solid-phase proximity ligation immunoassay. A PSA-binding peptide and an anti PSA MAb were conjugated with DNA probes and used to detect active PSA immobilized on a solid phase by another anti PSA MAb. This assay provided a 10-fold improvement in sensitivity but an even higher sensitivity is needed to analyze serum samples with low PSA concentrations. This proof of concept study shows that peptides reacting with proteins are potentially useful for sensitive and specific measurement of protein variants for which specific MAbs cannot be obtained.

4. REVIEW OF THE LITERATURE

4.1 Prostate cancer

4.1.1 Epidemiology

Prostate cancer (PCa) is the most commonly diagnosed non-skin cancer and second leading cause of cancer-related death of men in developed countries. Both incidence and mortality rates of PCa are highest in North America, and Northern and Western Europe, but much lower in Asia and Northern Africa (Jemal et al., 2006; Parkin et al., 2005; Postma & Schroder, 2005) (Fig. 1). It has been estimated in 2008 that PCa alone accounts for about 25% (186,320) of all newly diagnosed cancers and 10% (28,660) of all male cancer deaths in the USA (Jemal et al., 2008). In Finland the PCa incidence and mortality have been increasing since the 1960's (Fig. 2). The highest incidence was detected in 2006, when 4630 new cases of PCa were diagnosed. In 2007, 4189 new cases of PCa were reported, which corresponds to an incidence of 85.7 per 100,000 population. PCa ranks first of all male cancers, and accounts for 31.8% of new cancer cases in men; and it caused in 793 deaths, which corresponds to 13.8% of all causes of male cancer death, making PCa the second most common cause of male cancer death after lung cancer (Finnish Cancer Registry, 2009).

Incidence and mortality rates of PCa in black males is higher than that in white men, who have a higher rate than men of Asian origin in USA (Jemal et al., 2008). Differences in genetic variants (Corder et al., 1995; Devgan et al., 1997; Irvine et al., 1995; Platz et al., 2000; Shook et al., 2007), serum level of sex hormones (Ross et al., 1986; Ross et al., 1992; Winters et al., 2001; Wu et al., 2001a) and growth factors (Tricoli JV 1999, Platz EA 1999, Winter DL 2001) may contribute to racial differences in PCa incidence.

PCa is a slowly growing cancer (Carter et al., 1992a; Schmid et al., 1993; Stenman et al., 1999a), and incidence and mortality rates increase dramatically in men over 50 years of age (Fig. 3). In Finland, the median age of PCa at presentation is about 70 years while it is about 67.2 years in the USA (Finnish Cancer Registry, 2009; Shao et al., 2009).

Figure 1. Age-standardized incidence and mortality rates for PCa, per 100,000 men (Parkin et al., 2005). Reprinted with permission from John Wiley & Sons Inc.

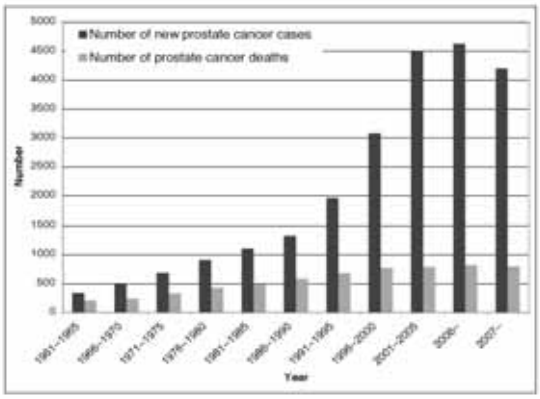

Figure 2. PCa Incidence and mortality in 1960-2007 in Finland (Finnish Cancer Registry, 2009).

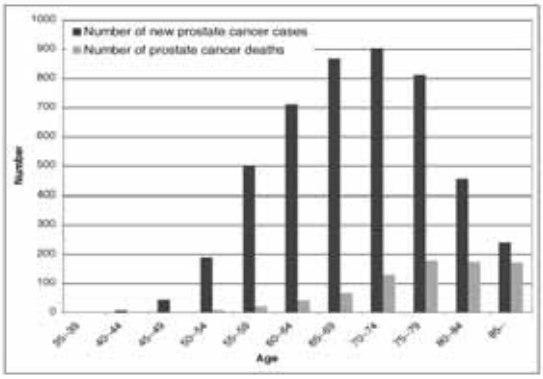

Figure 3. PCa Incidence rates and mortality rates by age in 2002-2007 in Finland (Finland Cancer Registry, 2009).

4.1.2 Risk factors

The etiology of PCa remains inconclusive. Many endogenous and environmental factors are linked to PCa risk, and some of them have been confirmed by epidemiologic studies (Bostwick et al., 2004; Schaid, 2004).

4.1.2.1 Genetic factors

Family history is associated with PCa risk (Ahn et al., 2008b; Hemminki & Dong, 2000; Negri et al., 2005). Men who have first-degree or second-degree relatives with PCa have an increased risk of PCa (Bruner et al., 2003; Johns & Houlston, 2003). Furthermore, there are ethnic differences in incidence and mortality in different populations (Jemal et al., 2008). This suggests that genetic factors play a critical role in PCa initiation and progression (Gronberg et al., 1994). A number of genetic alterations have been implicated in the development of PCa (Dong, 2006; Gsur et al., 2004; Schaid, 2004).

More recently, genome-wide association studies (GWAS) have provided a new approach to identify disease alleles. Large numbers of single nucleotide polymorphisms (SNPs) in the human genome have been analyzed to assess the association of the SNPs with PCa (Eeles et al., 2008; Witte, 2009; Zheng et al., 2008). Loci associated with PCa have been identified on chromosome 2q15 (Gudmundsson et al., 2008), chromosome 8q24

(Gudmundsson et al., 2007a; Haiman et al., 2007; Yeager et al., 2007; Zheng et al., 2008), chromosome 10 (Eeles et al., 2008; Thomas et al., 2008), and chromosome 17 (Gudmundsson et al., 2007b; Sun et al., 2008; Zheng et al., 2008). These loci contain some candidate susceptibility genes: β-microseminoprotein (*MSMB*) gene (Chang et al., 2009; Thomas et al., 2008), lemur tyrosine kinase 2 (*LMTK2*) gene (Eeles et al., 2008), kallikrein 3 *(KLK3)* gene (Ahn et al., 2008a; Eeles et al., 2008; Pal et al., 2007), Disabled homolog 2 interacting protein (*DAB2IP*) gene (Duggan et al., 2007), and hepatocyte nuclear factor 1 homeobox B (*HNF1B*) gene (Sun et al., 2008).

Recurrent chromosomal rearrangements may cause gene fusions. Recent experimental evidences suggest that gene fusions are key events driving the development and progression of PCa (Kumar-Sinha et al., 2008). In PCa, genomic rearrangements occur between the 5' untranslated end of transmembrane protease, serine 2 (TMPRSS2), a prostate-specific and androgen receptor-regulated gene, and the E26 transformation-specific family of genes (ETS family genes) that are oncogenic transcription factors (Tomlins et al., 2005). Of the *ETS* family, *ERG* (ETS-related gene) and *ETV1* (ETS-variant genes 1) are observed in about half of all PCa cases with *TMPRSS2-ETS* fusion (Hermans et al., 2006; Mehra et al., 2008; Perner et al., 2006; Tomlins et al., 2005).

4.1.2.2 Endogenous factors

Many endogenous, non-genetic factors also affect the development of PCa. These include hormones and growth factors. High levels of circulating testosterone and low levels of sex hormone-binding globulin are associated with increased risks of PCa (Gann et al., 1996). High testosterone concentrations in blood have been found to be associated with an increased risk for low grade PCa but with a reduced risk for high grade PCa (Platz et al., 2005; Schatzl et al., 2001). A higher ratio of testosterone to sex hormone–binding globulin is related to an increased risk primarily in men 65 years of age or older (Weiss et al., 2008). However, other studies find no relationship between PCa and sex hormones (Eaton et al., 1999; Roddam et al., 2008a).

Increased PCa risk has been reported to be associated with elevated plasma Insulin-like growth factor 1 (IGF-1) and decreased Insulin-like growth factor binding protein-3 (IGFBP-3) levels (Chan et al., 1998; Renehan et al., 2004; Roddam et al., 2008b; Stattin et al., 2000) although this is not found in other studies (Finne et al., 2000a; Weiss et al., 2007). Some studies indicate that IGF-1 is related to benign prostate hyperplasia (BPH) rather than PCa (Colao et al., 1999; Finne et al., 2000a).

PCa risk has been reported to be positively associated with body mass index (BMI) (Engeland et al., 2003; Rodriguez et al., 2001; Strom et al., 2008), waist to hip ratio (Hsing et al., 2000; Hubbard et al., 2004; Pischon et al., 2008), and high birth weight and length (Nilsen et al., 2005; Zuccolo et al., 2008). In contrast, men with diabetes have a lower risk of PCa (Bonovas et al., 2004; Leitzmann et al., 2008; Rodriguez et al., 2005). This may be associated with serum levels of sex hormones and growth factors (Buschemeyer & Freedland, 2007; Ding et al., 2006; Rogers et al., 2006).

4.1.2.3 Exogenous factors

There is large geographic variation in PCa incidence (Baade et al., 2004), and the incidence increases markedly in migrants who move from low risk countries to areas of higher risk (Beiki et al., 2008; Shimizu et al., 1991; Stellman & Wang, 1994; Yu et al., 1991). Some studies have suggested that exogenous factors are involved in the etiology of PCa

(Giovannucci et al., 2007). This is also supported by a twin study assessing the impact of heredity on cancer (Lichtenstein et al., 2000).

Diet Red meat consumption is positively associated with risk of PCa (Giovannucci et al., 1993; Koutros et al., 2008; Michaud et al., 2001; Schuurman et al., 1999), while consumption of fish may reduce the risk of PCa (Augustsson et al., 2003; Norrish et al., 1999; Terry et al., 2001). High consumption of vegetables, including cruciferous vegetables (Cohen et al., 2000; Jain et al., 1999; Kirsh et al., 2007), carrot (Kolonel et al., 2000), cabbage (Hebert et al., 1998), tomato (Bosetti et al., 2000; Giovannucci et al., 1995; Giovannucci et al., 2002) and soy (Kurahashi et al., 2007; Lee et al., 2003; Yan & Spitznagel, 2005), has been found to be associated with reduced PCa risk. Some studies show that the risk of PCa decreases with increasing consumption of green tea (Jain et al., 1998; Jian et al., 2004; Kurahashi et al., 2008), a high intake of vitamin E (Heinonen et al., 1998; Huang et al., 2003; Kirsh et al., 2006; Weinstein et al., 2005), and selenium intake (Etminan et al., 2005; Sabichi et al., 2006; van den Brandt et al., 2003).

Lifestyle Some studies have found that PCa incidence is positively associated with alcohol consumption (Middleton Fillmore et al., 2009; Platz et al., 2004; Sesso et al., 2001) and smoking (Gong et al., 2008; Malila et al., 2006; Plaskon et al., 2003; Sharpe & Siemiatycki, 2001). Epidemiologic evidence suggests that exposure to occupational agrochemicals (Alavanja et al., 2003; Strom et al., 2008; Van Maele-Fabry et al., 2006) and occupational chemicals (Agalliu et al., 2005; Krishnadasan et al., 2007; Rybicki et al., 2006) is related to increased PCa risk, while occupational physical activity is inversely associated with PCa incidence (Krishnadasan et al., 2008; Sass-Kortsak et al., 2007).

4.1.3 Diagnosis

Early detection and treatment of PCa may reduce mortality (Espey et al., 2007; Jemal et al., 2004; Martin et al., 2008). When detected at a localized stage, PCa is mostly curable, while survival is poor at the metastatic stage (Jemal et al., 2008). The American Cancer Society and the American Urological Association recommend that all men have yearly PCa screening beginning at 50 years of age (Bryant & Hamdy, 2008; Smith et al., 2008).

Primary care physicians have used digital rectal examination (DRE) to identify patients who need a prostate biopsy. DRE may eliminate unnecessary biopsies in selective screening procedures (Gosselaar et al., 2008b), but the procedure is not standardized and results vary widely (Kripalani et al., 1996; Phillips & Thompson, 1991), and the sensitivity and positive predictive value of DRE are low in patients with a serum PSA < 4 μg/L (Schroder et al., 2000; Schroder et al., 1998). Transrectal ultrasound (TRUS) provides a more precise estimate of prostate volume than DRE (Rietbergen et al., 1998). It is used to guide prostate biopsy and has greatly increased the diagnostic accuracy of this procedure (Berger et al., 2004; Gosselaar et al., 2008a; Uno et al., 2008).

Determination of PSA in serum has become the primary test for identification of men with increased risk of PCa since it was introduced more than 20 years ago. Increased PSA levels are associated with increasing risk of PCa, and it is less likely that the cancer will be curable when the PSA level is high (Hudson et al., 1989; Stamey et al., 1987; Stenman et al., 1994).

Prostate biopsy under TRUS guidance is used to detect PCa in men with increased PSA level or abnormal DRE. Sextant biopsy has been the standard procedure (Hodge et al., 1989), but recently, more biopsy cores are used to improve the diagnostic accuracy (Ravery et al., 2008; Scattoni et al., 2008; Singh et al., 2004). The more biopsy cores are taken, the more cancers will be found (Guichard et al., 2007).

4.1.4 Classification

The TNM staging system is used to classify solid tumors. T (tumor extent), N (regional lymph node status), and M (the presence or absence of distant metastasis) describe the extent of disease (Schroder et al., 1992; Sobin & Wittekind, 2002) (Table 1).

The Gleason grading system is the most commonly used for histopathological classification (Epstein et al., 2006; Gleason, 1966). Gleason grade is expressed by a score, which is assigned to cancerous tissue based on its microscopic appearance. The two most dominant patterns of cells are graded on scale of 1-5 and added together to determine the Gleason score. PCa mortality and tumor aggressiveness are strongly associated with the Gleason score (Albertsen et al., 1998). A Gleason score < 7 is associated with good prognosis while a score of 7 or higher indicates aggressive diseases.

Table 1. TNM classification for PCa (Frankel et al., 2003). Reprinted with permission from Elsevier.

Classification	Description
T1	**Not palpable or visible**
T1a	≤ 5% involved on a TURP sample
T1b	> 5% involved on a TURP sample
T1c	Needle biopsy positive (usually diagnosed because of high PSA)
T2	**Confined within prostate**
T2a	≤ half of one lobe
T2b	> half of one lobe
T2c	Both lobes
T3	**Outside prostate**
T3a	Extracapsular invasions
T3b	Seminal vesicle(s)
T4	**Fixed or invades adjacent structures**: bladder neck, external sphincter, rectum, levator muscles, pelvic wall
N	**Nodal status**
N0	No nodes
N1	Regional lymph node(s) positive
M	**Metastatic status**
M1a	Non-regional lymph node(s)
M1b	Bone(s)
M1c	Other site(s)

4.1.5 Biomarkers

A biomarker is a measurable indicator of a specific biological state, the presence and stage of disease. Biomarkers can be used clinically for screening, diagnosis and monitoring of disease activity to guide or assess therapy (Etzioni et al., 2003; Rifai et al., 2006). Prostatic acid phosphatase (PAP) was the first biomarker for PCa (Huggins, 1943). The PAP level in serum is elevated in metastatic PCa (Gutman & Gutman, 1938). It was widely used until PSA was shown to be more sensitive than PAP in the detection of PCa (Seamonds et al., 1986; Stamey et al., 1987).

PSA has been widely used as a PCa marker (details are introduced in next section). However, non-cancerous diseases may also cause elevation of serum PSA (Armitage et al., 1988; Guinan et al., 1987; Stamey et al., 1987). There is thus an urgent need for PCa biomarkers that can improve differentiation between benign and malignant disease, and detect potentially life-threatening tumors.

Many novel markers have been suggested as biomarkers for PCa (Table 2). Some of them have shown potentially clinical value. Human glandular kallikrein 2 (hK2, also called KLK2) is one of 15 members of the KLK family (Yousef et al., 2001), it shares about 80% identity at the amino acid and DNA level with PSA (also called KLK3) (Henttu & Vihko, 1989; Lundwall, 1989; Riegman et al., 1989b; Rittenhouse et al., 1998). Determination of serum hK2 may improve the specificity for detecting PCa (Becker et al., 2000; Darson et al., 1997; Steuber et al., 2007b). *TMPRSS2-ETS* fusion has been found in approximately 50% of PCa samples (Kumar-Sinha et al., 2008). Expression of the *TMPRSS2-ETS* fusion in prostate tissue is strongly associated with specific morphological features and adverse prognosis (Mosquera et al., 2007; Nam et al., 2007; Tomlins et al., 2005). Prostate cancer antigen 3 (PCA3) is a prostate-specific non-coding RNA. Recent studies have shown that the elevated PCA3 RNA level in urine is useful in the diagnosis for PCa (Bussemakers et al., 1999; van Gils et al., 2007). Serine peptidase inhibitor, Kazal type 1 (SPINK1), also known as tumor-associated trypsin inhibitor (TATI), is a specific inhibitor of trypsin. SPINK1 expression in tissue is found in high-grade PCa and this is associated with adverse prognosis (Paju A 2007, Tomlins SA 2008).

4.2 Prostate specific antigen (PSA)

In 1960 various prostate specific antigens were detected in prostatic tissue extracts (Flocks et al., 1960). In the 1970s several independent investigators subsequently identified and characterized a prostate specific protein in seminal plasma. This protein was called γ-seminoprotein (Hara et al., 1971), protein E (Li & Beling, 1973) or p30 (Sensabaugh, 1978). In 1979 Wang and coworkers purified a protein from prostate tissue and named it prostate specific antigen (PSA) (Wang et al., 1979). It is now generally accepted that γ-seminoprotein, protein E, p30 and PSA are the same protein (Wang et al., 1994). PSA was detected in serum from men with advanced PCa in the 1980's (Kuriyama et al., 1981; Papsidero et al., 1980). Since then PSA has become the most widely used serum marker for PCa (Catalona et al., 1991; Stamey et al., 1987).

Table 2. Potential biomarkers for PCa and their possible clinical utility.

Potential serum biomarkers	Possible Clinical utility	References
Chromogranin A (CgA)	Prognosis	(Berruti et al., 2000; Sciarra et al., 2008; Taplin et al., 2005)
Early prostate cancer antigen (EPCA)	Diagnosis	(Leman et al., 2007; Paul et al., 2005; Uetsuki et al., 2005)
Hepatocyte growth factor (HGF)	Diagnosis and Prognosis	(Gupta et al., 2008; Nagakawa et al., 2005; Naughton et al., 2001)
Huntingtin-interacting protein 1 (HIP1)	Diagnosis	(Bradley et al., 2005; Rao et al., 2002)
Insulin-like growth factor 1 (IGF-1)	Diagnosis	(Chan et al., 1998; Woodson et al., 2003)
Insulin-like growth factor binding protein-3 (IGFBP-3)	Diagnosis and Prognosis	(Chan et al., 1998; Shariat et al., 2002)
Interleukin-6 (IL6) and Interleukin 6 soluble receptor (IL6SR)	Diagnosis	(Nakashima et al., 2000; Shariat et al., 2001a)
hK2 (KLK2)	Diagnosis and Prognosis	(Darson et al., 1997; Partin et al., 1999; Steuber et al., 2007b; Wenske et al., 2009)
KLK11	Diagnosis	(Diamandis et al., 2002; Nakamura et al., 2003)
Macrophage inhibitory cytokine 1 (MIC-1)	Diagnosis and Prognosis	(Brown et al., 2006; Selander et al., 2007)
Osteopontin	Diagnosis and Prognosis	(Caruso et al., 2008; Fedarko et al., 2001; Hotte et al., 2002; Ramankulov et al., 2007)
Prostate secretory protein of 94 amino acids (PSP94)	Diagnosis and Prognosis	(Dube et al., 1987; Nam et al., 2006; Reeves et al., 2006)
Trefoil factor 3 (TFF3)	Diagnosis	(Faith et al., 2004; Garraway et al., 2004; Vestergaard et al., 2006)
Transforming growth factor-beta (TGF- $ß_1$)	Diagnosis and Prognosis	(Ivanovic et al., 1995; Shariat et al., 2001b; Truong et al., 1993)
Urokinase plasminogen activator (uPA)/Urokinase plasminogen activator receptor (uPAR)	Diagnosis and Prognosis	(Piironen et al., 2006; Shariat et al., 2007; Steuber et al., 2007a)

Table 2. Continue.

Potential urine biomarkers	Possible Clinical utility	References
Prostate cancer antigen 3 (PCA3)	Diagnosis	(Bussemakers et al., 1999; de Kok et al., 2002; Haese et al., 2008; van Gils et al., 2007)
Serine peptidase inhibitor, Kazal type 1 (SPINK1 or TATI)	Diagnosis	(Laxman et al., 2008; Paju et al., 2007; Tomlins et al., 2008)
Sarcosine	Diagnosis	(Sreekumar et al., 2009)

Table 2. Continue.

Potential tissue biomarkers	Possible Clinical utility	References
α-methylacyl-coenzyme A racemase (AMACR)	Diagnosis	(Luo et al., 2002; Rubin et al., 2002)
Annexin A3 (ANXA3)	Diagnosis	(Kollermann et al., 2008; Schostak et al., 2009)
Cysteine-rich secretory protein 3 (CRISP-3)	Diagnosis and Prognosis	(Asmann et al., 2002; Bjartell et al., 2006; Bjartell et al., 2007)
Enhancer of zeste homolog 2 (EZH2)	Diagnosis and Prognosis	(Bachmann et al., 2006; Laitinen et al., 2008; Varambally et al., 2002)
E-cadherin (ECAD)	Prognosis	(Ray et al., 2006; Rhodes et al., 2003)
Golgi phosphoprotein 2 (GOLPH2)	Diagnosis	(Kristiansen et al., 2008; Wei et al., 2008)
Glutathione S-transferases pi 1 (GSTP1)	Diagnosis	(Gonzalgo et al., 2003; Tokumaru et al., 2004)
Hepsin	Prognosis	(Dhanasekaran et al., 2001; Magee et al., 2001; Stephan et al., 2004)
Ki-67 protein	Prognosis	(Cowen et al., 2002; Goto et al., 2008)
p27	Prognosis	(Freedland et al., 2003; Thomas et al., 2000)
Phosphatase and tensin homologue (PTEN)	Prognosis	(Majumder & Sellers, 2005; McCall et al., 2008)
Proviral integration site for Molony murine leukaemia virus (PIM-1)	Diagnosis and Prognosis	(Dhanasekaran et al., 2001) (Cibull et al., 2006; Valdman et al., 2004)
Prostate-specific membrane antigen (PSMA)	Diagnosis and Prognosis	(Birtle et al., 2005; Bostwick et al., 1998; Ross et al., 2003)
Prostate stem cell antigen (PSCA)	Diagnosis	(Gu et al., 2000; Han et al., 2004)
TMPRSS2-ETS fusion	Diagnosis and Prognosis	(Mosquera et al., 2008; Nam et al., 2007; Tomlins et al., 2005)
Zinc-alpha2-glycoprotein (AZGP1or ZAG)	Diagnosis and Prognosis	(Hale et al., 2001; Henshall et al., 2006; Lapointe et al., 2004)

4.2.1 Expression

PSA is produced by the epithelial cells in both normal and neoplastic prostatic tissue. In the normal prostate, PSA is secreted into the lumen of the prostatic ducts (Qiu et al., 1990; Warhol & Longtine, 1985), while only small amount of PSA diffuse into circulation. Thus the concentration of PSA in seminal plasma is about one million-fold higher than in serum (Lilja, 1985). Due to disruption of the glandular architecture and loose contact with the prostatic ducts in PCa, PSA is secreted into blood, which results in elevation of serum PSA (Stenman et al., 1999a). The contribution of cancerous tissue to the serum levels of PSA is larger than that of normal prostatic and BPH tissue (Stamey et al., 1987) although PSA expression per cell is lower in carcinoma than in benign prostate epithelium (Pretlow et al., 1991), and it decreases with increasing Gleason grade (Abrahamsson et al., 1988; Aihara et al., 1994).

Elevated serum PSA concentrations are also related to prostatic volume (Babaian et al., 1990; Roehrborn et al., 1999). PSA expression is regulated by androgens (Henttu et al., 1992; Riegman et al., 1991). Thus androgen ablation, a standard therapy for advanced PCa (Sharifi N 2005), causes a rapid drop in serum PSA (Arai et al., 1990; Stamey et al., 1989).

PSA has been found at very low level in many tissues and body fluids (Shaw & Diamandis, 2007), and in some cancer tissues including lung, adrenal, kidney and colon (Levesque et al., 1995). The periurethral glands may produce PSA both in males and females (Iwakiri et al., 1993; Pollen & Dreilinger, 1984). In pregnant women amniotic fluid has been shown to contain low levels of PSA (Lovgren et al., 1999; Yu & Diamandis, 1995a). PSA is detectable in breast milk, cyst fluid and nipple aspirate fluid (Lovgren et al., 1999; Yu & Diamandis, 1995b). Clearly elevated PSA levels may occur in serum of females with breast cancer (Black et al., 2000; Diamandis, 2000; Melegos & Diamandis, 1996). However, in men PSA is in practice prostate specific.

4.2.2 Biochemistry

PSA (KLK3), also known as kallikrein-related peptidase 3, is a serine protease belonging to the KLK family. The *PSA* gene is located in chromosomal region 19q13.2-13.4 comprising all 15 KLK genes (Riegman et al., 1989a; Yousef & Diamandis, 2001). PSA is synthesized with a 17 amino acid leader sequence (preproPSA) that is cleaved cotranslationally to generate the inactive precursor protein (proPSA) containing 244 amino acids (Lundwall & Lilja, 1987; Mikolajczyk et al., 1997). Cleavage of the seven N-terminal amino acids from proPSA generates the active enzyme. Several prostatic proteases can activate PSA, including hK2 (Kumar et al., 1997; Lovgren et al., 1997; Takayama et al., 1997), trypsin (Paju et al., 2000; Takayama et al., 1997), KLK4 (Takayama et al., 2001b), and prostin/KLK15 (Takayama et al., 2001a; Yousef et al., 2001).

Mature PSA is a glycoprotein with a molecular weight of 28.430 kD, which contains 237 amino acids and one carbohydrate chain linked to asparagine 45 (Belanger et al., 1995; Lundwall & Lilja, 1987). The carbohydrate moiety is a biantennary complex oligosaccharide (Mattsson et al., 2008; Okada et al., 2001; Peracaula et al., 2003; Prakash & Robbins, 2000). Residues histidine 41, aspartate 96 and serine 189 form the active site of PSA. Active PSA has been shown to possess chymotrypsin-like substrate specificity (Watt et al., 1986). The best substrate for PSA contains the amino acid sequence SSIYSQTEEQ derived from the semenogelin sequence, in which PSA cleaves the peptide bond between Tyr (Y) and Ser (S) (Rehault et al., 2002).

The zinc concentrations in the prostate are high. Zn^{2+} at low micromolar concentration inhibits PSA activity (Hsieh & Cooperman, 2000; Malm et al., 2000; Watt et al., 1986). Two serine protease inhibitors (serpins), α1-chymotrypsin (ACT) (M_r of 68 kD) and α1-proteinase inhibitor (API) (M_r of 53kD), and the general protease inhibitor, α2-macroglobulin (A2M) (M_r of 725 kD) form complexes with PSA and inhibit the enzymatic activity of PSA in circulation.

4.2.3 Biological function

PSA is a major protein in seminal fluid and degrades the gel-forming proteins of semen, semenogelin 1 and 2. The physiological function of PSA is to liquefy the clot forming after ejaculation and cause release of motile spermatozoa (Lilja, 1985; Lilja et al., 1987). The

activity of PSA against commonly available peptide substrates is low, but it has been shown to cleave certain proteins in vitro. Cleavage by PSA results in inactivation of IGFBP-3, which may increase cell proliferation by releasing bound IGF-1 (Cohen et al., 1992). However, other prostatic proteases, like hK2 and trypsin, cleave IGFBP-3 more efficiently than PSA (Koistinen et al., 2002). PSA may promote cell migration and metastasis by cleaving fibronectin and laminin (Webber et al., 1995). PSA has also been shown to exert anti-angiogenic activity and it may thus slow down progression of PCa (Fortier et al., 1999). In vitro, PSA has been shown to convert plasminogen to biologically active angiostin-like fragments with anti-angiogenic activity (Heidtmann et al., 1999). The anti-angiogenic effect is dependent on the enzymatic activity of PSA. Internally cleaved and inactive PSA is devoid of this activity (Mattsson et al., 2008).

4.2.4 Molecular forms (Fig. 4)

A majority (60-95%) of the immunoreactive PSA in plasma is complexed with α1-chymotrypsin (PSA-ACT) (Lilja et al., 1991; Stenman et al., 1991). The PSA-α1-protease inhibitor complex (PSA-API) represents 1-10% of the total PSA immunoreactivity (Stenman et al., 1991; Zhang et al., 1999). PSA-α2-macroglobulin (PSA-A2M) cannot be detected by conventional immunoassays because PSA is engulfed by A2M, but it can be measured after PSA are released from the complex at a high pH. As measured by this method, PSA-A2M in serum represents 2-40% of total PSA (tPSA) (Zhang et al., 2000).

A variable part (5-40%) of PSA in plasma consists of various forms of free PSA (fPSA) that lack enzymatic activity due to nicking. Nicked PSA in serum is mature PSA which is internally cleaved between residues 85 and 86, 145 and 146 or 182 and 183 (Charrier et al., 1999; Hilz et al., 1999; Noldus et al., 1997). BPH tissue contains a distinct degraded form containing peptide bond cleavages at Lys145 and Lys182, which has been termed benign PSA (BPSA) (Mikolajczyk et al., 2000b).

A minor part of fPSA in plasma consists of proPSA, which lacks enzymatic activity and thus cannot form complexes with inhibitors (Lovgren et al., 1997; Vaisanen et al., 1999). proPSA represents 25-50% of fPSA in plasma (Mikolajczyk et al., 2004; Niemela et al., 2002)). It consists of (-7)proPSA (the full-length, 7 amino acid propeptide), and truncated forms (-5)proPSA (5 amino acid propeptide), (-4)proPSA (4 amino acid propeptide) and (-2)proPSA (2 amino acid propeptide) (Mikolajczyk et al., 2000a; Peter et al., 2001). Other truncated forms of fPSA, lacking a few amino acids at the beginning of N-terminus, have also been found in serum (Peter et al., 2001). Very low level of active PSA (3% of fPSA) has been observed in blood (Niemela et al., 2002).

In seminal plasma about 65% of PSA is enzymatically active and about 35% is nicked (Zhang 1995, Mattsson JM 2008).

4.2.5 Measurement of PSA

The first PSA immunoassay developed in 1980 was radioimmunoassay (Kuriyama et al., 1980). Currently available PSA assays are automated immunometric methods that facilitate sensitive, reliable and high-throughput analysis for screening, diagnosis, and monitoring of PCa. Recently, assays utilizing novel techniques, e.g., PCR or nanotechnology, have been developed. Some of them show higher sensitivity than conventional assays. Assays using

surface plasmon resonance (SPR) or microcantilevers facilitate rapid and easy determination, and show potential as point-of-care tests.

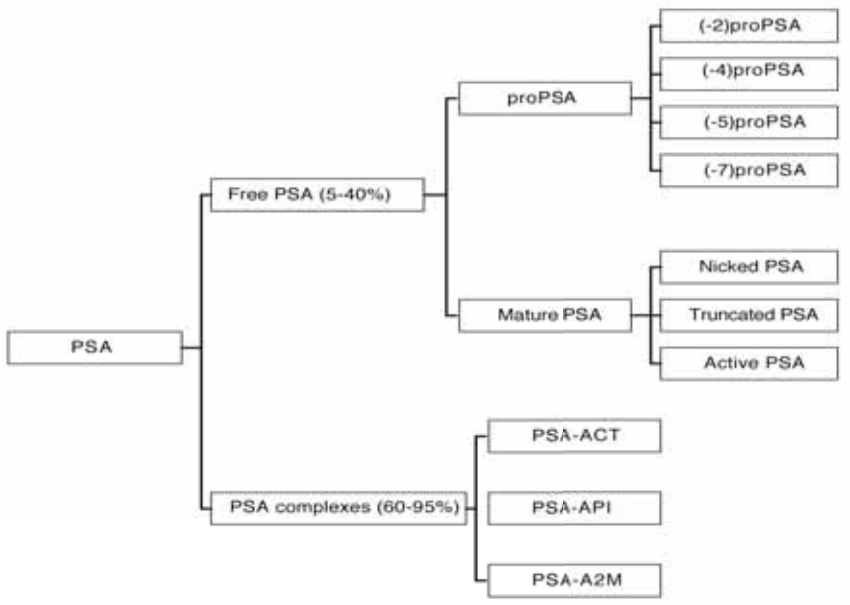

Figure 4. Molecular forms of PSA in blood.

4.2.5.1 PSA antibodies

Selecting an appropriate antibody pair is important for development of PSA immunoassays. Monoclonal antibodies (MAbs) against PSA have been classified into 6 major groups based on their epitopes (Stenman et al., 1999b). Group 1 antibodies are specific for fPSA, while group 2-6 antibodies react with both free and complexed PSA. Specific assays for various forms of PSA have been developed by combining different antibodies. However, some assays are not equimolar, i.e., they overestimate fPSA (Roddam et al., 2006; Zhou et al., 1993).

4.2.5.2 Standards

Standardization of PSA requires common standards. The First International Standards for tPSA (IRR 96/670) and fPSA (IRR 96/688) were established in 1999 (Rafferty et al., 2000). IRR 96/670 is a mixture of PSA and ACT in a 90:10 ratio mimicking circulating PSA, while IRR 96/688 contains fPSA. Use of these standards can help to reduce differences between assays (Stephan et al., 2006).

4.2.5.3 PSA immunoassays

The first commercial PSA assay (Pros-Check) was a traditional polyclonal radioimmunoassay (RIA) that was widely used in the early PSA studies (Yang Labortories, Bellevue, WA) (Stamey et al., 1987). The Hybritech Tandem-R PSA test is a sandwich-type

immunoradiometric assay, which is the first PSA test approved by the US Food and Drug Administration (FDA) (Hybritech, San Diego, CA). Most commercial assays are immunoenzymetric assays or immunochemiluminometric sandwich assays, and a majority of them use MAbs (Table 3). A time resolved immunofluormetric assay (IFMA), which uses antibodies labeled with stable fluorescent lanthanide chelates as detectors, measures fPSA and tPSA simultaneously (DELFIA PROSTATUS) (Perkin Elmer-Wallac, Turku, Finland).

Table 3. Characteristics of commercial PSA assay.

PSA assay	Parameter Assay type	Capture antibody	Tracer antibody	Signal molecule	Substrate	LDL (µg/L)
Yang Laboratories Pro-Check assay	RIA	P		^{125}I		0.25 (T)
Cis PSA-RICAT	IRMA	M	M	^{125}I		0.04 (T, F)
DPC IRMAcount	IRMA	M	M	^{125}I		0.1 (T)
Hybritech Tandem-R	IRMA	M	M	^{125}I		0.02 (T, F)
Abbott AxAYM	IEMA	M	M	ALP	mUP	0.04 (T), 0.02 (F)
Abbott IMX	IEMA	M	P	ALP	mUP	0.05 (T)
Bayer immuno-1	IEMA	M	P	ALP	pNPP	0.02 (T, C)
Backman ACCESS	IEMA	M	M	ALP	dP	0.008 (T), 0.005 (F)
CanAg EIA	IEMA	M	M	HRP	TMB	<0.1 (T, F)
DPC immulite	IEMA	P	M	ALP	dP	0.04 (T), 0.02 (F)
Hybritech Tandem-E	IEMA	M	M	ALP	pNPP	<0.1 (T)
Tosoh AIA-PACK	IEMA	M	M	ALP	mUP	0.05 (T)
Abbott ARCHITECT	ICMA	M	M	AE	H_2O_2	0.008 (T, F)
Bayer ACS:180	ICMA	M	P	AE	H_2O_2	0.09 (T)
Bayer ADVIA Centaur	ICMA	M	P	AE	H_2O_2	0.03 (C)
Perkin Elmer (Wallac) DELFIA	IFMA	M	M	Eu^{3+}, Sm^{3+}		0.1 (T), 0.04 (F)
Roche Elecsys	ECIA	M	M	ruthenium	tripropylamine	0.04 (T), 0.1 (F)

Abbreviations: LDL, lowest detection limit; RIA, radio immunoassay; IRMA, immunoradiometric assay; IEMA, immunoenzymetric assay; ICMA, immunochemiluminometric assay; IFMA, immunofluorometric assay; ECIA, electrochemiluminescent immunoassay; M, monoclonal; P, polyclonal; ALP, alkaline phosphatase; AE, acridinium ester; dP, dioxetane phosphate; HRP, horseradish peroxidase; ^{125}I , iodine-125; pNPP, para-nitrophenyl phosphate; TMB, tetramethylbenzidine; mUP, 4-methylumbelliferyl phosphate; Eu, europium; Sm, samarium; T, total PSA; F, free PSA; C, complexed PSA.

4.2.5.4 Immunoassay combined with polymerase chain reaction (PCR)

Immuno-PCR combining the specificity of antibodies with the sensitivity of PCR was first described in 1992 (Sano et al., 1992). A reporter system termed immuno-rolling circle amplification (RCA) has been used for sensitive detection of PSA (Schweitzer et al., 2000). An antibody-oligonucleotide conjugate binds to PSA that is captured on a solid surface by an

antibody. A DNA circle is hybridized to a complementary sequence in the oligonucleotide, and the DNA tag is amplified by RCA. The amplification results in a long DNA molecule, which contains hundreds of copies of the circular DNA sequence that remain attached to the antibody and that can be detected by fluorescent imaging. By this method, PSA has been detected at a concentration of 0.1 ng/L.

Real-time PCR uses measurement of fluorescence to monitor DNA amplification and quantitation of DNA concentration. In an immunoassay, PSA in the samples is captured by one antibody and detected by another antibody labeled with a DNA strand. Amplification of the DNA and measurement of PSA is performed by real time PCR. The detection limit is 4.8×10^5 PSA molecules in 5 µl (corresponds to 4.5 ng/L) (Link et al., 2004).

Proximity ligation assay is a novel, sensitive and specific method for quantitation of proteins (Fredriksson et al., 2002; Gullberg et al., 2004). About 300 PSA molecules in 5 µl (corresponds to 0.0028 ng/L) can be detected in a proximity ligation assay with triple-binders, which are a set of three proximity probes that recognize distinct epitopes on PSA (Schallmeiner et al., 2007).

4.2.5.5 Assays based on two-dimensional gel electrophoresis

Subforms of fPSA in serum can be separated and quantified by two-dimensional electrophoresis (Jung et al., 2004). PSA is extracted from serum by immunoadsorption and separated by two-dimensional electrophoresis. After blotted onto a membrane, PSA is detected with an antibody. The chemiluminescence intensities of the PSA spots are quantified with an image analyzer. PSA can be measured at concentration down to 0.1 µg/L (Jung et al., 2007; Tabares et al., 2007).

4.2.5.6 Immunoassay combined with mass spectrometry

PSA in serum was captured in 96-well microtiter plates with a monoclonal PSA antibody. Captured PSA is reduced, alkylated, and trypsin-digested in the wells, followed by extraction of the peptides on a C_{18} Ziptip. The peptides are analyzed on a linear ion-trap mass spectrometer and detected by product ion-monitoring mode. PSA has been detected at a concentration of 0.1 µg/L with a coefficients of variation (CV) < 20% (Kulasingam et al., 2008).

4.2.5.7 Assay based on surface plasmon resonance (SPR)

SPR can be used to measure proteins by detection of changes in mass concentration on a biospecific surface. A PSA-ACT assay based on SPR has been developed using an anti PSA-ACT antibody immobilized on the sensor surface. The detection limit is 18.1 µg/L for serum samples (Cao et al., 2006). The sensitivity of SPR-based assay for PSA has been improved by using an antibody-colloidal gold conjugate as a detector to amplify the SPR signal. A detection limit for PSA-ACT of 0.027 µg/L (Cao & Sim, 2007) and 0.15-1 µg/L for tPSA (Besselink et al., 2004; Huang et al., 2005) have been reported.

4.2.5.8 Assay based on surface plasmon field-enhanced fluorescence spectroscopy (SPFS)

SPFS, which combines SPR with sensitive fluorescence detection, uses the enhanced optical field of a surface plasmon mode at the metal-liquid interface to excite fluorescent molecules. In a SPFS-based assay, an anti PSA antibody is immobilized on the SPR sensor surface as a

capture antibody. A second anti PSA antibody labeled with fluorophores is used to detect PSA. A detection limit of 2 ng/L has been obtained (Yu et al., 2004).

4.2.5.9 Assays using an amperometric biosensor

PSA in samples is captured on an electrode surface containing glucose oxidase, and a PSA antibody-horseradish peroxidase (HRP) conjugate is used as a tracer. The concentration of PSA is determined by measuring changes in current caused by the enzymatic reaction of HRP. The limit of detection is 0.25 µg/L (Sarkar et al., 2002). In another PSA assay, Poly (1,2-diaminobenzene) is deposited on screen-printed electrodes to form an insulating layer. Polyaniline is electropolymerized in pores of the insulating layer produced by sonochemical ablation to form a microelectrode array. An anti PSA antibody is immobilized on conductive polyaniline protrusions. After binding of PSA to the antibody, alternating current impedance is used to measure the concentration of PSA. The detection limit of this assay is 1 ng/L (Barton et al., 2008).

4.2.5.10 Nanotechnology-based assays

Nanotechnology based on one-to-one interactions between analytes and signal-generating particles has shown potential utility in clinical diagnostics. PSA assays using nanotechnology have been developed and show high sensitivity (Table 4), e.g., immunoassays using fluorescent nanoparticles, composed of lanthanide chelates and polystyrene latex (Soukka et al., 2001), silica-coated material nanoparticles (Ye et al., 2004), or up-converting phosphor particles (UCP-particles) (Ukonaho et al., 2007), have shown to be 10-1000-fold more sensitive as compared to the conventional IFMA. Biobarcode is a nanoparticle probe composed of an oligonucleotide and an antibody. After PSA is bound to the magnetic microparticles, barcode DNA is dehybridized and amplified by PCR. A detection limit of 0.001 ng/L has been reported (Nam et al., 2003). However, the application for routine clinical use needs to be confirmed.

4.2.6 Clinical use of PSA

PSA has been widely used as a biomarker for PCa, both for diagnosis and monitoring of PCa, and it is considered the most useful of all existing tumor markers. A high PSA level in serum is usually associated with large tumor volume, high pathological stage and high Gleason grade (Antenor et al., 2005; Catalona et al., 1993; Catalona et al., 1991; Stamey et al., 1987).

4.2.6.1 Diagnosis of PCa

The serum concentration of PSA shows to increase in 5-10 years before a PCa is diagnosed on the basis of symptoms (Carter et al., 1992b; Stenman et al., 1994). It is therefore possible to detect PCa at a preclinical state by performing prostate biopsy in patients with an elevated serum PSA. FDA approved the PSA test as an aid for early PCa detection in 1994. Men are advised to have a biopsy when the PSA level in serum exceeds 4 µg/L (Catalona et al., 1994; Smith et al., 2006). The positive predictive value of a PSA level of above 4 µg/L is about 27% in the Europen screening studies (Hugosson et al., 2004; Makinen et al., 2004; Postma et al., 2007), and about 18% in screening trial in the USA (Andriole et al., 2005; Grubb et al., 2008).

Table 4. Assays based on nanotechnology.

Signal transduction	Nanoparticle or nanostructure	Lowest detection limit (ng/L)	Reference
Fluorescent nanoparticle	Silica-coated nanoparticle	7	(Ye et al., 2004)
	UCP-particle	0.53	(Ukonaho et al., 2007)
	Polystyrene latex nanoparticle	0.04	(Soukka et al., 2001)
Biobarcode	Gold nanoparticle	0.01	(Bao et al., 2006)
	Gold nanoparticle	0.001	(Nam et al., 2003)
SERS	Gold nanoparticle	1	(Grubisha et al., 2003)
SPR	Colloidal gold nanoparticle	<1000	(Huang et al., 2005)
	Colloidal gold nanoparticle	150	(Besselink et al., 2004)
	Colloidal gold Nanoparticle	27 (PSA-ACT)	(Cao & Sim, 2007)
Electrochemical signal	Quantum dot	200	(Wang et al., 2008)
	Quantum dot	20	(Liu et al., 2007)
	Gold nanoparticle	0.5	(Mani et al., 2009)
	Nanotube	250	(Okuno et al., 2007)
	Nanotube	4	(Yu et al., 2006)
	Nanowire	0.9	(Zheng et al., 2005)
	Microcantilever	1000	(Yue et al., 2008)
	Microcantilever	200	(Wu et al., 2001b)

Abbreviations: SERS, Surface-enhanced Raman scattering; SPR, Surface plasmon resonance; SPFS, surface plasmon field-enhanced fluorescence spectroscopy; UCP-particle, up-converting phosphor particles.

Approximately 75% of cancers are clinically organ-confined and potentially curable when PSA is in the range 4-10 µg/L (Catalona et al., 1994; Catalona et al., 1991; Labrie et al., 1992). Recent studies have shown that PCa can be detected in about 15% of men with PSA levels of less than 2 µg/L, and 15% of the cancers are high grade (Gleason grade ≥ 7) (Thompson et al., 2004). Some experts recommend biopsy for men with PSA above 2.5 µg/L, which significantly increases sensitivity (Catalona et al., 1997; Punglia et al., 2003).

In many developed countries the widespread use of PSA-testing has led to increased incidence of PCa with a decreased proportion of aggressive PCa (Aus et al., 2007; Laurila et al., 2009; Pelzer et al., 2008). However, the benefit of PCa screening is still debated (Bryant & Hamdy, 2008; Lin et al., 2008). PSA testing leads to overdiagnosis, i.e., the detection of cancer that otherwise would not have been diagnosed within the patient's lifetime (Schroder, 1995; Telesca et al., 2008). Overdiagnosis results in overtreatment, e.g., detection of insignificant cancers, creating patient anxiety and unnecessary costs (Draisma et al., 2003; McGregor et al., 1998). Results from two large randomized screening trials have recently been published. The US trial, the Prostate, Lung, Colorectal, and Ovarian screening trial (PLCO trial), is a multicenter, randomized, two-arm trial comprising 154 934 women and men aged 55–74 (Prorok et al., 2000). The European Randomized Study of Screening for Prostate Cancer (ERSPC) comprises approximately 193 000 recruited men from eight countries, which are randomly assigned to screening tests versus community patterns of care (de Koning et al., 2002). The results of ERSPC show that PSA screening reduces mortality from PCa, but 48 patients need to be treated in order to save the life of one patient (Schroder et al., 2009). In the PLCO trial, no reduction in mortality was observed (Andriole et al., 2009).

4.2.6.2 Monitoring of PCa after radical therapy

The FDA approved PSA as a marker to predict the risk of recurrence after treatment in 1986. A serum PSA of at least 0.4 µg/L and rising has been suggested as the standard for defining biochemical recurrence of PCa after radical prostatectomy (Stephenson et al., 2006a). Biochemical recurrence after radiation therapy has been defined as a PSA greater than the absolute nadir plus 2 µg/L or 2 consecutive increase of at least 0.5 µg/L (Horwitz et al., 2005).

4.3 Improving the clinical utility of PSA

PSA is not a cancer-specific biomarker. In addition to PCa, prostatitis, BPH and other conditions can also increase serum PSA (Stamey et al., 1987). Determinations of various forms of PSA and calculated parameters have been introduced to improve the diagnostic accuracy of the PSA test.

4.3.1 Various molecular forms of PSA in serum

In 1991 PSA was found to exist in different forms in serum, about 60-95% occurring as a complex with ACT, 5-40% being free and a small part being a complex with API. The proportion of PSA-ACT (%PSA-ACT) is higher and the proportion of fPSA (%fPSA) is lower in men with PCa than in those with BPH (Lilja et al., 1991; Stenman et al., 1991). Subsequent studies showed that a high %fPSA (e.g. >25%) is associated with reduced probability of PCa, while a low %fPSA (e.g. <10%) greatly increases the probability of PCa (Catalona et al., 1998; Catalona et al., 1995; Christensson et al., 1993). %fPSA is furthermore inversely correlated with tumor volume and Gleason score (Elgamal et al., 1996; Grossklaus et al., 2002; Southwick et al., 1999). FDA approved the fPSA test for PCa detection in 1998. Measurement of %fPSA increases the diagnostic accuracy at tPSA level both above 4 µg/L (Catalona et al., 1995; Luderer et al., 1995; Partin et al., 1996) and below 4 µg/L (Catalona et al., 1997; Finne et al., 2008; Walz et al., 2008).

The higher expression of ACT in malignant prostatic tissue (Bjork et al., 1994) and higher enzymatic activity of PSA secreted by PCa (Stenman et al., 1999a) can explain higher PSA-ACT level in serum from men with PCa than those without PCa. Determinations of PSA-ACT and %PSA-ACT improve the discrimination between PCa and BPH (Christensson et al., 1993; Kikuchi et al., 2006; Leinonen et al., 1993; Saika et al., 2002; Stenman et al., 1991). The proportion of PSA-API (%PSA-API) is higher in BPH than in cancer sera (Zhang et al., 1999). Measurement of %PSA-API has been shown to improve the clinical validity in PSA level above 4 µg/L. However, PSA-API cannot be reliably detected in samples with low PSA values (Finne et al., 2000b). Measurement of complexed PSA (cPSA), which includes PSA-ACT and PSA-API, has shown to improve the specificity of PCa (Allard et al., 1998; Miller et al., 2001; Okihara et al., 2006; Partin et al., 2003), but this has not been confirmed in other studies (Lein et al., 2003). This may be explained by the fact that the cPSA assay recognizes both PSA-ACT and PSA-API, which change in different directions in PCa (Stenman et al., 1991; Zhang et al., 1999).

PSA-A2M in serum cannot be detected by conventional immunoassays because A2M encapsulates PSA and hinders access of anti-PSA antibodies to PSA (Leinonen et al., 1996; Lilja et al., 1991). In a specific immunoassay, immunoreactive PSA is removed from serum

by immunoadsorption, and PSA-A2M is then denatured at high pH. The released PSA can be measured by a conventional PSA immunoassay. The proportion of PSA-A2M to tPSA is higher in patients with BPH than in those with PCa (Zhang et al., 1998). Measurement of the ratio of PSA-A2M to tPSA improves the diagnostic validity of PCa (Zhang et al., 2000).

proPSA represents about 30% of fPSA in PCa serum (Mikolajczyk & Rittenhouse, 2003). The (-2)proPSA level is higher in serum from patient with PCa than that with BPH (Chan et al., 2003; Mikolajczyk et al., 2001; Mikolajczyk et al., 2000a). Measurement of serum (-2)proPSA improves detection of PCa (Mikolajczyk et al., 2004; Sokoll et al., 2008). The ratio of proPSA to fPSA has been claimed to improve specificity for PCa detection in the PSA range 2 to 4 μg/L (Catalona et al., 2003; Sokoll et al., 2003). The level of BPSA in serum is associated with prostate volume (Naya et al., 2004) and is higher in BPH patients. The ratio of proPSA to BPSA improves detection of PCa in men with less than 15% fPSA (Khan et al., 2004). The concentration of intact PSA can be measured by an assay with a PSA antibody that does not recognize PSA nicked between amino acid 145-146. The ratio of intact PSA to fPSA is significantly higher in PCa than in BPH (Nurmikko et al., 2001; Steuber et al., 2002; Steuber et al., 2007a; Vickers et al., 2008). About 3% of fPSA in plasma is enzymatically active and thus only part of the active PSA in plasma forms complexes with serpins and A2M. Active PSA is of potential utility for detection of PCa (Niemela et al., 2002).

The carbohydrate structures in cancer cells are known to differ from those of nonmalignant cells, and this can be used for cancer diagnostics (Fukuda, 1996; Singhal & Hakomori, 1990). The glycan structures of PSA in serum, seminal plasma and LNCaP PCa cells are different (Ohyama et al., 2004; Okada et al., 2001; Peracaula et al., 2003; Prakash & Robbins, 2000). The content of α2,3-linked sialic acid in serum of PCa patients is lower than that in seminal plasma (Tabares et al., 2006) and potentially discriminates malignant from benign conditions (Tajiri et al., 2008). Reduced sialylation of tPSA is found in PCa, and measurement of PSA with α2,6-linked sialic acid in serum with a lectin immunosorbant assay improves detection of PCa compared to %fPSA (Meany et al., 2009).

4.3.2 Age specific reference ranges

Serum PSA level increases with age, presumably due to the increasing incidence of BPH among elderly men (Babaian et al., 1992; Verhamme et al., 2002). Age-specific reference ranges have been proposed to increase the sensitivity of detection in younger men and the specificity in older men (Table 5) (Morgan et al., 1996; Oesterling et al., 1993; Oesterling et al., 1995). However, this approach has been criticized for missing clinically significant cancers in older men (Borer et al., 1998; Catalona et al., 1994).

4.3.3 PSA kinetics

The long-term change in serum PSA is termed PSA velocity. An increase of serum PSA of more than 0.75 μg/L per year is associated with increased risk of PCa (Carter et al., 1992a), and PSA velocity is useful for PCa detection (Carter & Pearson, 1993; Loeb et al., 2007; Punglia et al., 2007). PSA velocity can also indicate tumor aggressiveness and risk of mortality after radiation therapy (D'Amico et al., 2004; D'Amico et al., 2005; Loeb et al., 2008; Partin et al., 1994; Robinson et al., 2008), but this has not been confirmed in other studies (Pinsky et al., 2007; Ulmert et al., 2008).

Table 5. Reference ranges for free, complexed and total PSA, and free-to-total, complexed-to-total and free-to-complexed PSA ratios according to patient age (Oesterling et al., 1995). Reprinted with permission from Elsevier.

Parameter	Age Group (yrs.)			
	40 to 49	50 to 59	60 to 69	70 to 79
Serum PSA concentrations (ng./ml.):				
Free	0.5	0.7	1.0	1.2
Complexed	1.0	1.5	2.0	3.0
Total	2.0	3.0	4.0	5.5
PSA ratios:				
Free-to-total	⇐ More than 0.15 ⇒			
Complexed-to-total	⇐ Less than 0.70 ⇒			
Free-to-complexed	⇐ More than 0.25 ⇒			

For free, complexed and total PSA the upper limit of normal was defined as the 95th percentile for the midpoint of age range from regression analysis. For the free-to-total, complexed-to-total and free-to-complexed PSA ratios 1 reference range is applicable to all age groups, since the ratios are not dependent on patient age.

PSA doubling time (PSADT) is defined as the time needed for the PSA value to double. PSADT is shorter in men with than without PCa (Raaijmakers et al., 2004; Spurgeon et al., 2007). A short PSADT following radiation therapy and radical prostatectomy is correlated with tumor aggressiveness (Leibman et al., 1998; Pruthi et al., 1997; Teeter et al., 2008).

The PSA concentrations in serum are influenced by individual biological variation, and measurement of PSA velocity and PSADT requires several determinations over a long time (Ornstein et al., 1997; Roehrborn et al., 1996; Soletormos et al., 2005). Optimal number and time of individual measurements are not known. Furthermore, differences between different PSA assays can result in an artificial PSA change (Link et al., 2004; Stephan et al., 2006). Therefore the use of PSA kinetics is controversial (Ramirez et al., 2008; Vickers et al., 2009).

4.3.4 PSA density

PSA density is the ratio of serum PSA to prostate volume estimated by TRUS. The PSA density is higher in men with PCa than in those with prostatitis and BPH (Benson et al., 1992; Kundu et al., 2007; Rietbergen et al., 1998; Veneziano et al., 1990). Some studies have reported that the transition zone volume more accurately predicts a positive biopsy than total prostate volume (Djavan et al., 1998; Kalish et al., 1994; Zlotta et al., 1997). However, this has not been confirmed in other studies (Brawer et al., 1993; Lin et al., 1998; Ohori et al., 1995). Furthermore, the limited accuracy of TRUS affects the calculation of PSA density (Matlaga et al., 2003).

4.3.5 Statistical and mathematical methods

Multivariate analysis can be used to explain the relationship between diagnostic variables and the outcome variable. Different multivariate logistic regression (LR) models and artificial

neural networks (ANNs) have been developed to improve diagnostic accuracy for PCa detection (Carlson et al., 1998; Djavan et al., 2002; Finne et al., 2001; Kawakami et al., 2008; Stephan et al., 2005; Virtanen et al., 1999). ANNs can also predict the stage of PCa (Han et al., 2001; Tewari, 1997; Zlotta et al., 2003) and biochemical failure after radical prostatectomy (Potter et al., 1999; Poulakis et al., 2004; Ziada et al., 2001). This facilitates estimation of the need for adjuvant therapy (Potter et al., 1999; Poulakis et al., 2004; Ziada et al., 2001). The variables used include various molecular forms of PSA, clinical stage, age and prostate volume.

A model using PSA concentration, clinical TNM stage, and biopsy Gleason score, called "Partin tables," is widely used to predict pathological stage of PCa (Partin et al., 1997; Partin et al., 1993). Nomograms based on PCa biomarkers, clinical stage, and Gleason grade have been developed to predict PCa relapse after radical prostatectomy (Kattan et al., 1998; Kattan et al., 1999; Shariat et al., 2008; Stephenson et al., 2006b).

5. AIMS OF THE PRESENT STUDY

The aim of the present study was to develop specific assays for detection of various forms of PSA in circulation and to evaluate their validity for diagnosis of prostate cancer.

The specific aims were:

1. To develop a novel monoclonal antibody and an immunoassay for PSA-ACT (I).
2. To develop novel immunoassays using a peptide specifically reacting with active free PSA (II, III).
3. To develop a sensitive immunoassay for PSA-API based on proximity ligation (IV).

6. MATERIALS AND METHODS

6.1 Serum samples (I, II, IV)

Serum samples from men with or without evidence of cancer (I, II, IV), and from healthy females (I, II, IV) were obtained from the Department of Clinical Chemistry, Helsinki University Central Hospital. Serum samples (IV) were obtained from 139 men participating in the Finnish Part of the European Randomized Screening for Prostate Cancer (Maattanen et al., 2001). All patient samples were taken before initiation of therapy, and frozen once and stored at -80°C until analyzed. The diagnoses were based on histological analysis of tissue obtained by biopsy or at surgery. Human seminal fluid was obtained from Department of Obstetrics and Gynecology, Helsinki University Central Hospital.

6.2 Antibodies

The antibodies used are listed in Table 6.

6.2.1 Development of MAbs (I, IV)

BALB/c mice were immunized with 10–30 µg of PSA-ACT or PSA-API by intraperitoneal injection with Freund's complete adjuvant. A booster dose of 10 µg was administered after 4 weeks, with additional boosters of 100 and 150 µg administered 1 and 2 days after the first booster dose, respectively. After the final booster, the splenic lymphoid cells of the mice were fused with the mouse myeloma cell P3x63-Ag8.653 (American Type Culture Collection). The fused cells were harvested in HAT medium supplemented with interleukin-6 for 4 weeks. Antibody production was evaluated by an in-house IFMA. MAbs were purified from cell culture fluid medium by protein G affinity chromatography (Amersham Pharmacia Biotech AB, Uppsala, Sweden).

Table 6. List of antibodies used.

Antibody	Specificity [1]	Source [2]	Used in
5A10	Anti fPSA	H. Lilja	III
9B10	Anti fPSA	H. Lilja	III
4G10	Anti fPSA	J. Leinonen	II
5C7	Anti fPSA and PSA-ACT	J. Leinonen	III
2E9	Anti fPSA and PSA-ACT	Perkin Elmer-Wallac	III
E91	Anti fPSA and PSA-ACT	E. Paus	III
H50	Anti fPSA and PSA-ACT	Abbott	III
9C5	Anti fPSA and PSA-ACT	J. Leinonen	III
5E4	Anti fPSA and PSA-ACT	J. Leinonen	II, III, IV
1D10	Anti complexed ACT	L. Zhu	I
8G8	Anti API	L. Zhu	IV
Rabbit anti PSA	Anti fPSA and PSA-ACT	DAKO	I
Rabbit anti ACT	Anti ACT	DAKO	I
Rabbit anti API	Anti API	DAKO	IV

[1] Details described in (Becker et al., 1999)
[2] Details described in (Stenman et al., 1999b) and this thesis.

6.3 Chromatographic methods

6.3.1 Gel filtration (I, II, IV)

Gel filtration was performed on a 1.6 x 60 cm Superdex-200 column (Amersham Pharmacia Biotech) equilibrated with 50 mmol/L Tris-HCl buffer (pH 7.7) containing 150 mmol/L NaCl (Tris-buffered saline, TBS). The flow rate was 15 mL/h, and 2-mL fractions were collected into tubes containing 200 µL of TBS with 5% bovine serum albumin (BSA) (Sigma, St. Louis, MO).

6.3.2 Ion exchange chromatography

6.3.2.1 Cation exchange chromatography (II, III)

The sample diluted 10-fold with 50 mmol/L phosphate buffer, pH 5.6 (buffer A) was applied to a 1-ml Resource S column (Amersham Pharmacia Biotech) equilibrated with buffer A. Proteins were eluted with a linear gradient composed of 60 ml of buffer A and 60 ml of buffer A containing 0.5 mol/L NaCl.

6.3.2.2 Anion exchange chromatography (I, IV)

A 1-ml Resource Q column (Amersham Biosciences) was equilibrated with 10 mmol/L Tris-HCl buffer containing 8 mmol/L sodium azide, pH 8.4 (buffer B). The serum sample was diluted 10-fold volume of buffer B and applied to the column. Proteins were eluted with a linear gradient composed of 60 ml of buffer B and 60 ml of buffer B containing 0.3 mol/L NaCl.

6.3.3 Affinity chromatography (II, III)

The sample was applied to an immunoaffinity column prepared by coupling a monoclonal anti-PSA antibody (5E4 or 4G10) to CNBr-activated Sepharose (Amersham Pharmacia Biotech). The column was washed with 30 bed volumes of 50 mmol/L Tris buffer containing 0.5 mol/L NaCl (pH 8) or 50 mmol/L phosphate buffer (pH 5.6). Protein was eluted with 0.1% trifluoroacetic acid and neutralized with 1 mol/L Tris base.

6.4 Production and purification of proteins

6.4.1 Purification of PSA from seminal plasma (II)

Seminal plasma was precipitated with ammonium sulfate at 25% and 70% saturation. The precipitate obtained with 70% saturation was diluted and applied to an affinity chromatography column with MAb 5E4. PSA was further purified by ion-exchange chromatography on a Resource Q column.

6.4.2 Purification of proPSA from LNCaP cell medium (II, III)

proPSA was purified from LNCaP cell culture medium by immunoaffinity chromatography with anti-PSA MAb 4G10. Affinity-purified LNCaP PSA was further separated by ion exchange chromatography on a Resource S column (Amersham Pharmacia Biotech). The fraction containing proPSA was activated by treatment with bovine trypsin (Sigma).

6.4.3 Preparation of PSA-ACT, PSA-API and cathepsin G-ACT (I, IV)

PSA-API, and PSA-ACT were prepared by incubating purified PSA with API (Athens Research and Technology Inc, Athens, GA), or ACT (Sigma) at 37 °C for 48 h. PSA-API or PSA-ACT were further separated by gel filtration on a Superdex-200 column (Amersham Pharmacia Biotech). The cathepsin G-ACT complex was prepared by incubating purified cathepsin G with ACT at 37 °C for 30 min.

6.5 PSA-binding peptides (II, III)

PSA-binding peptides were identified by screening phage display peptide libraries (Koivunen et al., 1993; Koivunen et al., 1994; Wu et al., 2000). Two peptides, B-2 (CVFAHNYDYLVC) and C-4 (CVAYCIEHHCWTC), were constructed as GST fusion proteins, expressed in Escherichia coli BL21 cells and purified by glutathione affinity chromatography (Amersham Pharmacia Biotech) as described previously (Wu et al., 2000).

6.6 Labeling and biotinylation of proteins

Antibodies and other proteins were labeled with either Eu^{3+} or Sm^{3+} chelates of N1-(p-isothiocyanatobenzoyl)-diethyleneamine-N1,N2,N3,N3-tetraacetic acid according to the manufacturer's instructions (Perkin Elmer-Wallac, Turku, Finland) (I, IV).

Biotinylation of antibodies with Sulfo-NHS-LC-biotin was performed according to the manufacturer's instructions (Pierce, Rockford, IL) (III, IV).

6.7 Measurement of enzymatic activity (II)

Purified proPSA (100 ng) was incubated in microtiter wells coated with monoclonal antibody 5E4 for 1 h at 22 °C with slow shaking. After washing of the wells twice, bound proPSA was activated by addition of 10 ng of bovine trypsin (Sigma) in 200 µL of buffer (5% BSA in TBS) and incubated for 20 min at 22 °C. After washing, a fluorescent peptide substrate (Denmeade et al., 1997) (Enzyme Systems Products, Livermore, CA) was added to a final concentration of 400 µmol/L, and the absorbance (excitation wavelength at 355 and emission wavelength at 460 nm) was monitored for 120 min in a Victor 1420 Multilabel counter (Perkin Elmer-Wallac).

6.8 Measurement of fPSA and tPSA (I, II, III, IV)

fPSA and tPSA were measured by a dual-label IFMA. Twenty five µL of standards from the ProStatus PSA fPSA/tPSA kit (Perkin Elmer-Wallac) and samples together with 200 µL of assay buffer (Perkin Elmer-Wallac) were added to microtiter plate wells coated with MAb H117 or MAb 5E4. After 1 h incubation at room temperature with slow shaking, the plates were washed twice. 200 µL of assay buffer containing Eu^{3+}-labeled MAb 5A10 and Sm^{3+}-labeled MAb H50 (Perkin Elmer-Wallac) were added. After incubation for 2 h at room temperature with shaking, unbound antibodies were removed by washing four times. 200 µL of enhancement solution (Perkin Elmer-Wallac) was added and the fluorescence was measured after incubation for 5 min. All samples were tested in duplicate.

6.9 Immunoblotting (IV)

SDS-PAGE was performed in 2-mm thick, 3-16% gradient polyacrylamide gels. Proteins were transferred electrophoretically to nitrocellulose membrane and incubated either with MAb 5E4 or a MAb against API (Table 6). Bound antibodies were detected with horseradish peroxidase-conjugated polyclonal rabbit anti mouse IgG (DAKO, Glostrup, Denmark) using 3,3'-diaminobenzidine tetrahydrochloride as a substrate.

6.10 Construction of proximity probes (III, IV)

Proximity probes with either free 3' or 5' end were prepared by conjugating thiol-modified oligonucleotides to maleimide-derivatized streptavidin (Sigma). The conjugates were subsequently reacted with biotinylated antibodies or biotinylated peptides as described (Gullberg et al., 2004). The oligonucleotide sequences were as follows:
5' free STV, 5'P-
TCGTGTCTAAAGTCCGTTACCTTGATTCCCCTAACCCTCTTGAAAAATTCGGCATC
GGTGA-3';
3' free STV, 5'-
CGCATCGCCCTTGGACTACGACTGACGAACCGCTTTGCCTGACTGATCGCTAAAT
CGTG-3'OH.

6.11 Assay development

6.11.1 Time-resolved IFMAs for fPSA/tPSA and the PSA-ACT complex (I)

Sera with high PSA concentrations were pooled and used as a calibrator (the concentration range of the calibrators was 0.8-120 µg/L calculated on the basis of tPSA). Calibrators or serum samples (25 µL) were added to wells coated with MAb H117, followed by 100 µL of assay buffer (Perkin Elmer-Wallac). After incubation for 1 h at room temperature with shaking, the wells were washed twice. In the double-label assays for PSA-ACT and tPSA, 200 µL of assay buffer containing tracer antibodies, Eu^{3+}-1D10 and Sm^{3+}-H50, were added together. However, in the double-label assay for fPSA and PSA-ACT, there was interference between the Sm^{3+}-1D10 and the Eu^{3+}-5A10. If they were added simultaneously or the Sm^{3+}-

1D10 was added first and the Eu^{3+}-5A10 1 h later, there was 10-fold increase in the background of the fPSA assay. This problem was eliminated when the Eu^{3+}-5A10 was added first and incubated for 1 h before addition of Sm^{3+}-1D10. After further incubation for 2 h and washing four times, 200 µL of enhancement solution (Perkin Elmer-Wallac) was added to the wells. After shaking for 5 min at room temperature, the fluorescence was measured with a Victor 1420 Multilabel counter (Perkin Elmer-Wallac).

6.11.2 Immunopeptidometric assay (IPMA) (II)

Pure enzymatically active PSA was used as a calibrator. 25 µL of samples and calibrators in 200 µL of assay buffer [50 mmol/L Tris-HCl (pH 7.7), 150 mmol/L NaCl, 67 µmol/L BSA, 1 µmol/L bovine IgG] were added to MAb 5E4-coated wells and incubated for 1 h at 22 °C. After incubation, the wells were washed, and 1 µg of GST fusion protein in 200 µL of assay buffer was added. After incubation for 60 min at 22 °C, the wells were washed, and 50 ng of Eu^{3+}-labeled anti-GST antibody (Amersham Pharmacia Biotech) in 200 µL of assay buffer was added and incubated for 60 min. The wells were washed four times, and enhancement solution (Perkin Elmer-Wallac) was added. Slow shaking was used during all incubations. After 5 min, time-resolved fluorescence was measured.

6.11.3 Immunoassay based on proximity ligation (III, IV) (Fig. 5)

In the assay, 1 µg of monoclonal anti-PSA antibody 5E4 in 50 µl of PBS was allowed to adsorb to ABI PRISM optical tubes (Applied Biosystems, Foster City, CA) overnight at 4 °C. Unadsorbed antibodies were washed away with PBS and the non-specific binding sites of the wells were saturated with assay buffer (1% BSA in PBS) overnight at 4 °C. 50 µL of standards or samples in duplicate were added to the tubes. After incubation for 1 h at room temperature, the tubes were washed twice with PBS and 50 µL of assay buffer and proximity probes (100 pmol/L each) were added. After incubation for 2 h at room temperature, unbound probes were removed by washing twice with 200 µL of Milli-Q water. The ligation and TaqMan PCR mix (50 µL) (Gullberg et al., 2004) was added. Oligonucleotide sequences were as follows: Connector oligonucleotide, 5'-TACTTAGACACGACACGATTTAGTTT-3'; forward primer, 5'-CATCGCCCTTGGACTACGA-3'; reverse primer, 5'-GGGAATCAAGGTAACGGACTTTAG-3'. After ligation for 5 min at room temperature the tubes were transferred to a real-time PCR instrument for temperature cycling at 95 °C for 2 min, followed by 95 °C for 15 s and 60 °C for 60 s, repeated 40 times (Applied Biosystems PRISM 7000). The ligation and TaqMan PCR mix used in the study III were provided by Olink AB (Uppsala, Sweden).

6.12 Statistical analyses

The detection limits for assays were calculated from the mean concentration plus two standard deviations of the signal in female sera with unmeasurable PSA concentrations (I, IV) or in assay buffer (II, III). Pearson parametric correlation coefficients were used to describe the correlation between different assays (I). The correlation between the IFMA and proximity ligation assay was determined by linear regression analysis (IV). Differences in

concentrations of various forms of PSA between PCa patients and controls were calculated with the Mann-Whitney *U*-test (I, IV). Differences in specificity at various sensitivities were compared by the McNemar test (I, IV). Receiver operation curve (ROC) analysis was used to estimate the diagnostic validity of various tests (I, IV). Statistical analysis was performed with SPSS 15.0 for Windows (SPSS. Inc., Chicago, IL).

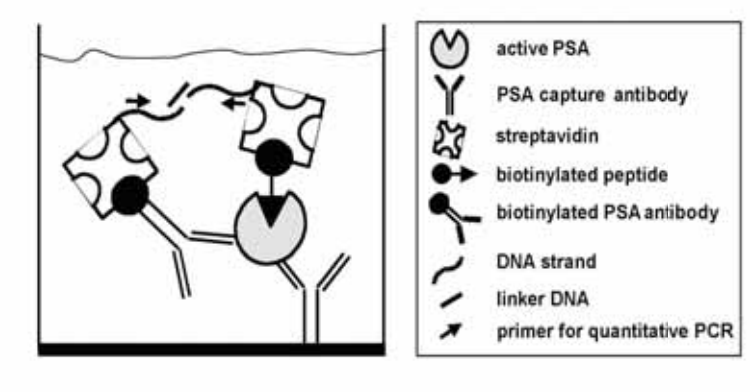

Figure 5. Principle of proximity ligation assay.

7. RESULTS

7.1 Characterization of MAbs

7.1.1 Development of MAbs to ACT (I)

Three MAbs (1D10, 8D2, and 1E2) reacted more strongly with PSA-ACT than with free ACT, while they did not react with fPSA or PSA-API. These MAbs recognized the captured cathepsin G-ACT complex. Of these, MAb 1D10 showed the lowest reactivity with free ACT and cathepsin G-ACT as compared to PSA-ACT (Fig. 6). MAb 1D10 was selected as a tracer in the PSA-ACT assay.

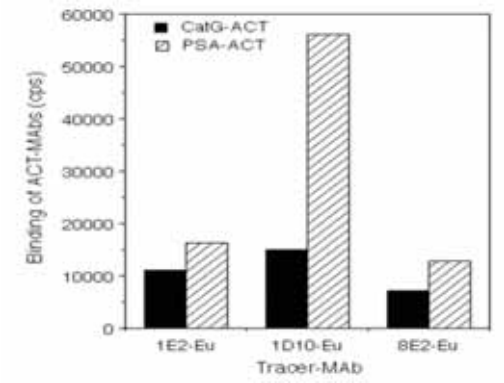

Figure 6. Reactivity of anti-ACT MAbs with cathepsin G-ACT (CatG-ACT) and PSA-ACT complexes.

7.1.2 Development of MAbs to API (IV)

Four MAbs (8G8, 8E8, 2E11 and 13D10) reacted with both API and PSA-API, but not with PSA. This was confirmed both by Western immunoblotting (Fig. 7) and by IFMA using Eu^{3+}-labeled PSA and API. MAb 8G8 showed the highest affinity and was selected for the PSA-API assay. The specificity of MAb 8G8 was further tested with purified fPSA, hK2, PSA-API, API, PSA-ACT and trypsin-2-API in an IFMA using MAb 5E4 as a capture antibody. The results showed that the combination of MAb 5E4 and Eu^{3+}-labeled MAb 8G8 recognized only PSA-API.

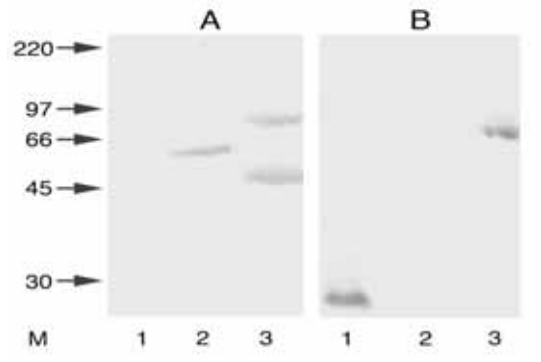

Figure 7. Immunoblotting with anti-API MAb 8G8 (A) and anti-PSA MAb 5E4 (B). Lane M, molecular mass markers; Lane 1, PSA; Lane 2, API; Lane 3, PSA-API.

7.2 Assay performance

7.2.1 Dual-label immunoassay for measurement of PSA-ACT with either fPSA or tPSA (I)

In the dual-label assay for PSA-ACT and fPSA, the detection limit was 0.05 µg/L for PSA-ACT and 0.004 µg/L for fPSA, respectively. In the dual-label assay for PSA-ACT and tPSA, the detection limits were 0.03 µg/L for PSA-ACT and 0.02 µg/L for tPSA. The intra-assay coefficients of variation (CVs) and inter-assay CVs were from 0.4% to 6.1% for all assays (Table 7). The mean analytic recovery of fPSA was 93.3% (range, 80.6-106.5%), and that of PSA-ACT was 99% (97.5-102.8%), respectively.

For fPSA, the correlation between the double-label assays for fPSA/PSA-ACT and fPSA/tPSA was: $y = 1.15x - 0.13$ ($r = 0.98$). The correlation between PSA-ACT concentrations measured by the fPSA/PSA-ACT and PSA-ACT/tPSA assays was: $y = 1.03x + 0.15$ ($r = 0.99$). The calculated concentrations of tPSA based on the sum of fPSA and PSA-ACT obtained with the fPSA/PSA-ACT assay and those measured by the fPSA/tPSA and PSA-ACT/tPSA assays also correlated well ($y = 1.07x - 0.27$; $r = 0.99$ and $y = 1.07x - 0.29$; $r = 0.99$).

Table 7. Precision and reproducibility of double-label assays for tPSA/fPSA and PSA-ACT.

	CV%				
	tPSA	**fPSA**	**PSA-ACT**	**PSA-ACT /tPSA**	**fPSA/ PSA-ACT**
Dual-label assays for PSA-ACT/tPSA					
Sample 1 (5.3 µg/L)					
Intra-assay	2	1.5	2.2	0.8	2.9
Inter-assay	3.2	3.9	3	0.4	3.6
Sample 2 (13.5 µg/L)					
Intra-assay	3.1	3.1	3.8	0.4	6.1
Inter-assay	2.7	4.1	2.3	0.7	4.8
Dual-label assay for fPSA/PSA-ACT					
Sample 1 (5.3 µg/L)					
Intra-assay	2.5	2.6	3.2	1.9	4.6
Inter-assay	3.5	3.5	3.6	3.5	2.1
Sample 2 (13.5 µg/L)					
Intra-assay	3	3.3	3.2	2.4	3.2
Inter-assay	3	2.3	3.6	1.2	5.2

7.2.2 IPMA for enzymatically active PSA (II)

The detection limit of the IPMA was 0.6 µg/L. The intra- and interassay CVs of the assay were 7-12% and 8-13%, respectively, at concentrations of 2-100 µg/L. The reactivities of the PSA isoenzymes (A-E), which display variable enzyme activities, were analyzed by the IPMA. GST-peptides B-2 and C-4 bound equally to the intact isoenzymes A and B (defined as 100%), whereas the response was 25%, 15%, and 7% with PSA isoenzymes C, D, and E, respectively (Fig. 8). proPSA purified from LNCaP cell culture medium showed low enzymatic activity. After activation by trypsin, the activity increased. Approximately 6-9% of proPSA was recognized by the IPMA, and after activation with trypsin recognition increased

to 71-88% compared with that of PSA-B.

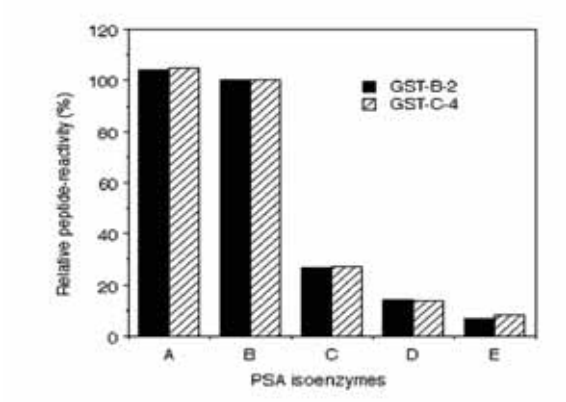

Figure 8. Reactivity of various PSA isoenzymes in the IPMA.

7.2.3 Proximity ligation assay for active PSA (III)

The detection limit of the assay was 0.07 µg/L and the dose response was linear up to a concentration of 25 µg/L. The inter-assay CVs were 23.5% and 19.6%, and intra-assay CVs were 21.7% and 14.4% at PSA concentrations of 0.56 and 3.18 µg/L, respectively. The assay did not cross-react with proPSA or with hK2.

7.2.4 Measurement of the complex between PSA and API by proximity ligation (IV)

The analytical detection limit was 6.6 ng/L. The intra-assay CVs were 18.3 and 17.8% and the inter-assay CVs 20.6 and 19.0% in serum with PSA-API concentrations of 0.25 and 1.22 µg/L, respectively.

Pure PSA-API was diluted to concentrations of 0.02-4.98 µg/L with 1% BSA in PBS and measured by IFMA and by the proximity ligation assay for PSA-API. The correlation between the results was good ($y=1.04x+0.13$, $R^2 = 0.98$).

One µg of pure PSA-B was added to 0.5 ml of female serum. After incubation for 48 h, the sample was subjected to anion exchange chromatography. The fractions were analyzed for fPSA and tPSA with an IFMA and PSA-API with proximity ligation assay. A minor peak containing PSA-API was separated from the major peaks containing fPSA and PSA-ACT (Fig. 9).

7.3 Molecular forms of PSA in serum

7.3.1 PSA-ACT (I)

Sera from patients with or without PCa with PSA concentrations in the range 2-20 µg/L were measured with dual-label IFMA. The median concentration of PSA-ACT was 3.67 µg/L in patients without PCa and 3.93 µg/L in PCa men, while the proportion of fPSA to PSA-ACT

(%fPSA/PSA-ACT) was 31.6% and 24.8%, respectively. The apparent mean value of PSA-ACT in 84 female sera was 0.02 µg/L, which is below the detection limit of the assay. None of 84 values exceeded the detection limit.

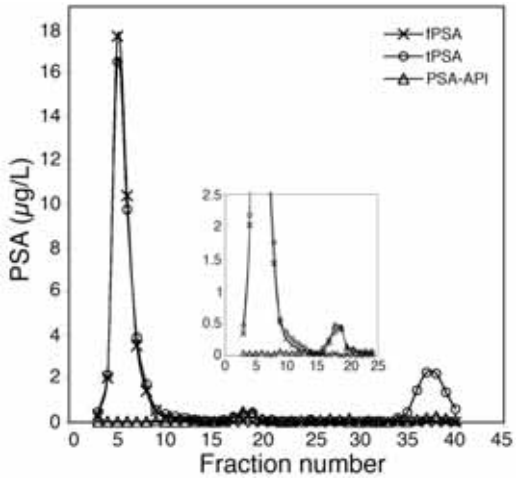

Figure 9. Separation of three major forms of PSA in plasma by anion-exchange chromatography.

7.3.2 Active PSA (II)

Active PSA in serum samples containing above 10 µg/L fPSA was analyzed by IPMA. The concentrations measured corresponded to 1–10% of the fPSA concentration. No PSA was detected by the IPMA in 10 female sera.

7.3.3 PSA-API (IV)

Samples with tPSA concentrations of 4.01-9.78 µg/L were used to evaluate the validity of PSA-API. The median concentration of PSA-API was 0.17 µg/L in screening negative men and 0.16 µg/L in men diagnosed with PCa, while the median %PSA-API was 3.3% (range, 0.38-14.4%) in screening negative men and 2.8% (range, 0.23-13.4%) in PCa cases.

7.4 Clinical validity of PSA complexes

7.4.1 PSA-ACT (I)

The area under the curve (AUC) for %fPSA/PSA-ACT was higher than those for either tPSA or PSA-ACT alone, and their difference was highly significant (P <0.001). There were no significant differences in AUC values between %fPSA, %PSA-ACT, and %fPSA/PSA-ACT. The AUC values for PSA-ACT alone or the calculated values for complexed PSA (tPSA minus fPSA) were not significantly larger than those for tPSA (Fig. 10).

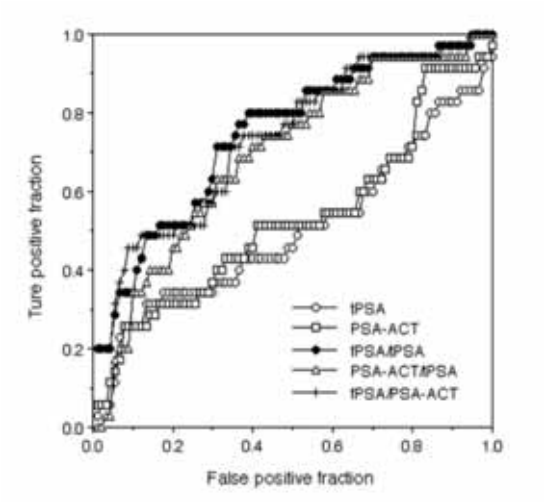

Figure 10. ROC curves for individual analytes and ratios based on 35 PCa and 90 control samples with PSA concentrations of 2-20 µg/L.

7.4.2 PSA-API (IV)

The AUC for %PSA-API was 0.546 while that for tPSA was 0.536. The AUC value of %fPSA was 0.710, which was significantly greater than that of tPSA (P= 0.011). Because both %fPSA and %PSA-API were lower in cancer cases than in controls, we calculated whether the sum of them provided further improvement. The AUC value for sum of %fPSA plus %PSA-API was 0.723, which was slightly larger than that for %fPSA alone and significantly larger than that for tPSA (P =0.006).

8. DISCUSSION

PCa is the most common cancer and the second leading cause of cancer-related deaths in Western men. Early detection and treatment of PCa is thought to reduce mortality of PCa (Espey et al., 2007; Jemal et al., 2004; Martin et al., 2008). Because the serum concentrations of PSA increase about 5-10 years before PCa surfaces clinically, it is possible to detect PCa at an early and potentially curable stage (Gann et al., 1995; Stenman et al., 1994). Thus PCa is a potential screening target. However, the first results from PCa screening studies show that the reduction of mortality is small (Schroder et al., 2009) or not significant (Andriole et al., 2009). Thus, better screening strategies and tumor markers are needed.

PSA is organ-specific but not cancer-specific, and therefore benign prostatic diseases can also cause an increase of PSA in circulation. Thus only about one third of patients with moderately elevated PSA level (4-10 μg/L) have a positive biopsy while the rest are false positives (Andriole et al., 2005; Catalona et al., 1998). On the other hand, the biopsy results may be false negative in cases of small local cancer, especially if only a limited number of biopsy cores are taken. Improved diagnostic accuracy of the PSA test is therefore needed, and this can be achieved by measurement of various molecular forms of PSA (Lilja et al., 2008). Measurement of PSA-ACT, fPSA, PSA-API, PSA-A2M, enzymatically active PSA and proPSA have all been shown to improve the clinical validity of PSA, and assays for fPSA are widely available. However, assays for many other forms are hampered by various technical problems. Thus, the assays for PSA-ACT and PSA-API tend to have a high nonspecific background, while assay of enzymatically active PSA has been limited by lack of specific antibodies (Stenman et al., 1991; Zhang et al., 1997).Various methods have previously been used to reduce the background in assays for PSA-ACT, e.g., addition of milk casein (Wu et al., 1998) and heparin into the assay buffer (Pettersson et al., 1995) or use of a PSA-ACT-specific MAb (Wang et al., 1999). In a previous assay for PSA-API, the non-specific background was measured in a well coated with an unrelated antibody and the fluorescence in this well was subtracted from that in the corresponding assay well (Finne et al., 2000b). However, these methods were only partially successful. An alternative approach is to measure these two complexed forms of PSA together in an assay for complexed PSA (cPSA) (Allard et al., 1998). This assay measures PSA-ACT and PSA-API together, which is a principal disadvantage because PSA-ACT increases while PSA-API decreases in PCa.

8.1 Dual-label immunoassays for PSA-ACT and fPSA/tPSA (I)

Measurement of PSA-ACT and calculation of %PSA-ACT improves the discrimination between PCa and benign prostatic disease compared with tPSA (Lilja et al., 1991; Stenman et al., 1991) but previously available assays were hampered by a background caused by nonspecific binding to the solid phase of a large excess of uncomplexed ACT and other ACT-protease complexes (Leinonen et al., 1993). To reduce this problem we developed MAbs that preferentially detected PSA-ACT but not free ACT or other ACT complexes. Antibody 1D10 showed good specificity and dual label assays with this MAb as a tracer were developed to simultaneously measure PSA-ACT and tPSA or fPSA. By these assays PSA-ACT was undetectable in female sera showing that the problem with nonspecific background had been eliminated. The correlation between PSA-ACT measured with dual assay for PSA-ACT and tPSA or dual assay for fPSA and PSA-ACT was excellent. An advantage of the dual assay is that tPSA and PSA-ACT, or fPSA and PSA-ACT are measured in the same assay, and the imprecision caused by pipetting errors is reduced which improves measurement of the ratios.

ROC analysis revealed that %PSA-ACT gives the same discrimination between cancers and controls as %fPSA. All calculated ratios, i.e., %fPSA/PSA-ACT, %PSA-ACT, and %fPSA, provided the same improvement in cancer specificity. This finding confirms that the assays and the calculated ratios perform as theoretically expected.

8.2 Proximity ligation assay for PSA-API (IV)

Assay of PSA-API is complicated both by the high non-specific background and by the low concentrations of PSA-API in serum (Zhang et al., 1999). Proximity ligation assay has been shown to be an ultrasensitive and highly specific method for detection of proteins (Fredriksson et al., 2002; Gullberg et al., 2004). The assay uses a pair of DNA probes bound to matched antibodies. When these antibodies bind to a target protein, the ends of the oligonucleotides are brought into proximity and can be hybridized with a probe that is complementary to the antibody bound oligonucleotides. The DNA strand formed in the ligation reaction is amplified and quantified by a real time PCR. The need for binding of both probes in close proximity to each other can be expected to prevent detection of API nonspecifically bound to the solid phase and this should reduce assay background. Furthermore, DNA amplification increases assay sensitivity.

We therefore decided to develop a sensitive and specific immunoassay for PSA-API based on proximity ligation. To improve sensitivity we developed a specific MAb to API (8G8) and used it as a probe. In the assay, PSA-API was captured to the solid phase by a MAb against tPSA and detected by two biotinylated MAbs, MAb 8G8 and MAb against tPSA, which were bound to streptavidin carrying the DNA probes. Simultaneous binding of these MAbs to the PSA-API complex brought the DNA strands close to each other allowing their ligation and thereafter amplification by real time PCR. The detection limit of assay was 0.64 ng/L, which is about 10-fold better than in a previous IFMA (Finne et al., 2000b). The use of 3 rather than 2 antibodies has been shown to enhance specificity and sensitivity also in other proximity ligation assays (Gustafsdottir et al., 2006; Schallmeiner et al., 2007). The concentrations of PSA-API in female sera were undetectable, showing that the problem with nonspecific background caused by adsorption of API to the solid phase had been eliminated. PSA-API could thus be detected in plasma with PSA concentrations of 4-10 µg/L.

Correlation between results obtained by IFMA and by the proximity ligation assay was good and separation of various PSA complexes by ion exchange chromatography showed that the new assay specifically recognized PSA-API. The imprecision of our assay was higher than that of conventional immunoassays, which has been reported for other immunoassays utilizing PCR amplification (Lind & Kubista, 2005; Niemeyer et al., 2007).

Serum samples from PCa patients and controls with tPSA concentrations of 4-10 µg/L were used to evaluate the clinical utility of PSA-API. As shown in our earlier study, %PSA-API tended to be lower in sera from men with PCa than in controls. The AUC value for %PSA-API improved discrimination between cancer cases and controls as compared to tPSA, but %fPSA was clearly superior to %PSA-API. However, the effect of %fPSA and %PSA-API was additive and the diagnostic validity was improved by calculating the sum of these two variables. The improvement was modest but it was similar to that observed in our earlier study (Finne et al., 2000b) and similar to the improvement obtained by combining %fPSA with assay of hK2 (Steuber et al., 2007b).

8.3 Assays for enzymatically active PSA (II, III)

Enzymatically active free PSA is of potentially diagnostic value (Niemela et al., 2002), but we have not obtained any antibody that specifically recognizes active PSA. We therefore developed an immunopeptidometric assay using as a tracer a peptide binding to active PSA (II). A MAb against tPSA was used to capture all isoforms of PSA and active PSA was detected with peptides that were expressed together with GST as fusion proteins (Wu et al., 2000). Bound peptide was detected by time-resolved fluorometric assay with a Eu^{3+} labeled anti GST antibody. The assays, using peptides B-2 and C-4, detected the various seminal plasma PSA isoenzymes with variable enzymatic activities to an extent corresponding to their enzymatic activities. proPSA was not recognized by this assay, but after activation with trypsin it reacted in the assay. The detection limit of the assay, 0.6 µg/L, enabled measurement of active PSA in samples with elevated PSA concentrations.

Fractionation of serum with a high PSA concentration by gel filtration and measurement of the fractions by the IPMA showed that approximately 10% of fPSA was enzymatically active. Furthermore, direct assay of sera from PCa patients showed that 1–10% of fPSA was enzymatically active in spite of the large excess of protease inhibitors in plasma. Previously about 3% of fPSA in circulation has been found to be enzymatically active (Niemela et al., 2002). This study shows that peptides can be used together with antibodies for development of novel immunoassays, but the IPMA is not sensitive enough for measurement of active PSA in sera with the moderately increased concentrations that are typically present at early stages of PCa.

To increase assay sensitivity, we developed an immunoassay for active PSA based on proximity ligation (III). A biotinylated peptide recognizing active PSA and a MAb against tPSA were used to construct a pair of probes, which detected active PSA captured by another MAb to tPSA. The detection limit of this assay was 0.07 µg/L, which was ten-fold better than that of the IPMA. hK2, which has about 80% amino acid sequences identity with PSA, and proPSA were not detected with this assay. Thus this assay is of potential utility for evaluation of the utility of active PSA for differentiation between PCa and benign prostatic diseases.

In comparison with other proximity ligation assays with very high sensitivity (Gullberg et al., 2004; Schallmeiner et al., 2007), the relatively moderate improvement in the sensitivity of this assay was likely due to the low affinity of the peptide for PSA, i.e., 100–1000-fold lower than that of high-affinity antibodies. It has been shown that probe affinity and assay sensitivity are strongly correlated (Gullberg et al., 2004). Like in the PSA-API assay, the precision of the assay was inferior to that of conventional immunoassays.

A number of detection technologies have been used to develop very sensitive assays for PSA and other proteins occurring at very low concentrations, i.e, in some cases in the range 0.001 ng/L (Table 4). Most of these require the use of custom built instruments and unique chemicals, whereas assay based on proximity ligation uses readily available instruments and chemicals. Proximity ligation has been used to establish an extremely sensitive assay for fPSA, but we did not achieve a similar sensitivity for PSA-API and enzymatically active PSA. However, the assays developed had other unique properties that have not been obtained before with other approaches. These properties can be utilized to develop other assays for problematic analytes.

9. CONCLUSIONS

We developed novel immunoassays for measurement of PSA-ACT and PSA-API in serum with low PSA levels. Measurements of these together with fPSA and %fPSA improve the diagnostic accuracy for PCa. IPMA is a new assay principle, by which enzymatically active PSA could be detected by utilizing a PSA-binding peptide. Assays using peptides with unique selectivity are potential tools for measurement of proteins for which antibodies cannot be obtained. Proximity ligation can be used to improve sensitivity of conventional assays, and this principle is also useful for reduction of the unspecific background.

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