Prostate-specific Antigen: Its Usefulness in Clinical Medicine

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Prostate-specific antigen (PSA) was discovered about 20 years ago and over the last decade it has become the premier tumor marker for diagnosis, monitoring and prognosis of prostatic carcinoma. PSA is now considered to be the best tumor marker available in clinical medicine. It is the only tumor marker that has been approved by the Food and Drug Administration of the USA for mass screening: for the purpose of diagnosing early prostatic carcinoma. Among the newest developments in the field are the discovery of the molecular forms of PSA in serum, the development of ultrasensitive assays that allow better monitoring of patients after radical prostatectomy, and the discovery of non-prostatic PSA. Indeed, there are indications that PSA might be useful for the diagnosis and prognosis of breast cancer. The genomic structure of PSA and other human kallikrein genes and the regulation of their expression has recently been elucidated. Currently, the PSA promoter and enhancer are being investigated in connection with gene therapy in prostatic tissue. Efforts are now underway to supplement the clinical value of PSA measurements with additional prostatic markers, including human kallikrein 2 (hK2) and prostate-specific membrane antigen (PSMA).

Prostate-specific antigen (PSA) is a 30-kDa glycoprotein produced primarily by the prostatic epithelial cells and secreted into the seminal plasma where it is present at relatively huge concentrations (~0.5–3 mg ml^{-1}). Much lower concentrations (~10^6 times lower) are found in the serum of normal males. Interest in this molecule has increased dramatically over the last ten years primarily because of its usefulness as a screening marker for prostatic adenocarcinoma. The number of papers per month dealing with PSA between the years 1980–85, 1986–91 and 1992–96 were one, nine and 28, respectively (Chu 1997). Despite the extensive literature, this field of investigation is still growing rapidly. In this review, I will briefly cover some basic knowledge on PSA and describe its present position as a tool in clinical medicine.

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• Historical Perspective

Several investigations reported the production of antibodies against unpurified prostatic antigens in the 1960s. Because these antigens were not characterized definitively, none of these investigators can be credited with the discovery of PSA (Armbruster 1993). The search for the presence of male-specific antigens in semen, for forensic purposes, led to the discovery of three 'prostate-specific' antigens: gamma-seminoprotein (Hara *et al.* 1971), protein E (Li and Beling 1973) and protein p30 (Sensabaugh 1978). Later studies indicated that these three proteins were all PSA (Graves *et al.* 1990). Definitive characterization of PSA was accomplished in 1979 (Wang *et al.* 1979). PSA was identified in the serum of prostate cancer patients in 1980, and the first enzyme-linked immunosorbent assay (ELISA) of this antigen became available shortly afterwards (Chu 1997). In 1986, the PSA blood test was approved by the Food and Drug Administration (FDA) for prostate cancer monitoring, and in 1994 the FDA approved the test for the early detection of prostate cancer (population screening), in combination with digital-rectal examination (DRE). The PSA test is the only blood test that has been approved by the FDA for the early detection of any cancer.

• PSA Structure and Function

The PSA gene is a member of the human kallikrein gene family, which comprises PSA and another two genes, *hKLK1* and *hKLK2* (McCormack *et al.* 1995). The gene for PSA is also known as human kallikrein 3 (*hKLK3*). All three genes are clustered within a 60-kb genomic region on chromosome 19q13.3–q13.4. The coding and promoter–enhancer regions of the PSA gene have been well characterized. The PSA gene has five exons and encodes a 237-amino acid mature protein, which is secreted. The PSA protein is glycosylated at a single site (asparagine 45). PSA gene regulation is mediated through the steroid hormone receptor system. The PSA gene promoter–enhancer region spans approximately 5 kb of DNA and contains three distinct androgen response elements (AREs) (Schuur *et al.* 1996, Cleutjens *et al.* 1997b). Androgens (and possibly progestins and glucocorticoids) upregulate gene expression dramatically, in a tissue-specific manner (Cleutjens *et al.* 1997b).

PSA is a serine protease with chymotrypsin-like enzymatic activity. PSA acts upon semenogelin I, semenogelin II and fibronectin in seminal plasma, causing liquefaction of the seminal plasma clot after ejaculation (Lilja 1987). PSA might have other potential biological functions, including growth factor regulation, both within and outside the prostate (Peehl 1995). Recently, this suggestion has been strengthened by the demonstration of increased serum insulin-like growth factor 1 (IGF-1) levels in individuals at high risk for developing prostate cancer (Chan *et al.* 1998).

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• **Molecular Forms of PSA in Serum**

Studies of seminal plasma indicate that most of the PSA in this fluid is present in its 30-kDa form. This 'free PSA' fraction is not homogeneous and comprises both enzymatically active (50–70%) and enzymatically inactive PSA (30–50%) (Zhang *et al.* 1995). The enzymatically inactive form of PSA has internal peptide-bond cleavages that render the protease inactive. Other possible free forms of inactive PSA include the proenzyme form, non-clipped but denatured PSA and a +1PSA form, which is missing the N-terminal isoleucine (Rittenhouse *et al.* 1998).

In serum, three major PSA fractions have been identified (Lilja *et al.* 1991, Stenman *et al.* 1991). The complex of PSA and α_2-macroglobulin cannot be measured by current ELISA-type assays for PSA and its diagnostic value is unknown. The complex of PSA and α_1-antichymotrypsin (PSA-ACT) comprises ~80% (common range, 45–95%) of the serum immunoreactive PSA and has a molecular mass of ~100 kDa. The remainder of the serum immunoreactive PSA has a molecular mass of ~330 kDa and is known as free PSA. Free PSA is considered biologically inactive but its structure is unknown. It might represent just one or a mixture of the inactive PSA forms found in seminal plasma or an inactive zymogen form (Mikolajczyk *et al.* 1997). The assay of the various forms of PSA and their clinical usefulness is described below.

• **PSA Assays: Standardization**

The PSA molecule has six major antigenic sites. One antigenic site is masked completely upon binding of PSA to ACT, making it very useful for designing assays that can measure free PSA specifically. For assays that measure total serum PSA, any of the appropriate remaining five epitopes can be used. Early assays that measure PSA with competitive-type radioimmunoassay (RIA) principles are now rarely used. Currently, most assays use 'sandwich-type' configurations, based on either two monoclonal antibodies or one monoclonal and one polyclonal antibody. There are no known crossreacting compounds except for the highly homologous hKLK1 and hKLK2 proteins. With appropriate selection of monoclonal antibodies, the crossreactivity of these antigens can be minimized.

Historically, kits from various manufacturers have produced different results, making clinical decisions and comparisons difficult. The recent efforts to epitope map several anti-PSA antibodies (Corey *et al.* 1997a, Wang *et al.* 1998) and to standardize calibration of commercial kits (Stamey 1997) are already producing better agreement among different assays. Current assays for total PSA (free PSA plus PSA-ACT) should recognize free PSA and PSA-ACT with the same potency (equimolarity) and should be free of crossreactivity with hKLK1 and hKLK2, and current free PSA assays should be free of crossreactivity with PSA-ACT.

Current commercial kits have detection limits around 0.1 $\mu g\ l^{-1}$. More recently, increased emphasis has been given to designing assays with a 10–100-fold better sensitivity. Such 'ultrasensitive' or 'third-generation' assays are more useful as monitoring tools (Ferguson *et al.* 1996). It is expected that over the next 2–3 years many commercial kits for monitoring will have detection limits around 0.01 $\mu g\ l^{-1}$, or lower.

• **PSA-based Screening for Prostate Cancer Detection**

Prostate cancer is a very prevalent disease, and it is now the most frequently diagnosed cancer in males. It is estimated that in the USA alone, a new diagnosis of prostate cancer is made every 3 min. Contrary to widely perceived notions, prostate cancer is an aggressive disease that kills many people; of all patients afflicted, 25% die because of the disease. The death rate from prostate cancer in the USA is about one every 15 min. Because there is currently no way to prevent this cancer, efforts to diagnose and treat prostate cancer early are gaining much attention and support.

Prostate cancer is most prevalent in older men, and efforts to diagnose it early are currently focusing on men 50 years of age, or older. Physicians can detect prostate cancer by performing DRE. This procedure is not definitive and a suspicious DRE must be accompanied by transrectal ultrasonography (TRUS) and biopsy to confirm the diagnosis. It is known that DRE misses a significant number of cancers even when performed by experienced urologists.

Although, at first glance, screening for prostate cancer might look a very attractive strategy for early diagnosis and treatment (Brawer *et al.* 1993, Catalona *et al.* 1993), this has been, and still is, a subject of controversy. In Table 1 we present the most frequently asked questions about screening and offer short answers based on literature reports. Clearly, definitive answers to the critical question of extended patient survival with screening must await completion of prospective clinical trials now in progress.

• **Staging and Prognosis**

Staging is defined as the anatomical extent of the disease. For prostate cancer, relevant information for staging includes extracapsular extension, seminal vesicle involvement, positive surgical margins, positive lymph nodes and distant metastatic sites. A number of techniques have been used to predict the anatomical extent of the disease, including computerized tomography (CT) scans, magnetic resonance imaging (MRI) scans, radionuclide bone scan, pedal lymphangiography, pelvic lymph node dissection, biopsy, Gleason grade and pre-operative PSA. Obviously, some of these techniques are very expensive and others have significant morbidity. On the other hand, accurate pathological staging is important and can guide the physician in selecting the best possible treatment modality. Unfortunately, PSA analysis alone is not a good predictor of staging. However, Narayan *et al.* (1995) showed that biopsy-based stage, pre-operative serum PSA and Gleason score provide a good prediction of final pathological stage. While very high pre-operative PSA ($>100\ \mu g\ l^{-1}$) is indicative of advanced metastatic disease, Oesterling *et al.* (1993) found that a pre-operative PSA of $10\ \mu g\ l^{-1}$ or less is almost never associated with a positive bone scan. This finding supports the elimination of the

Table 1. Questions and answers related to PSA population screening

1) Q: What is the additional benefit of adding the PSA test to DRE in screening for prostate cancer?
   A: The cancer detection rate is increased by about 50%. The combination of the two tests is a more sensitive and specific index than either test alone.
2) Q: What is the prostate cancer detection rate of PSA screening in asymptomatic individuals over the age of 50?
   A: 3–5% in Americans and Europeans; 1% in Japanese.
3) Q: Is the incidence of prostate cancer rising?
   A: The recent surge in incidence rates (1990–1995) is due to the introduction of PSA screening programs. Incidence rates have now been stabilized or are even decreasing.
4) Q: What is the recommendation of the American Cancer Society and American Urologic Association regarding screening?
   A: PSA and DRE should be part of the annual check-up in men over 50 years of age. If there is a family history of prostate cancer or are African–American, annual check-ups should start at age 45 years. Elevated PSA (>4 $\mu g\ l^{-1}$) should be further investigated by TRUS and biopsy.
5) Q: Does PSA screening detect early, localized disease?
   A: Yes. Since the PSA test has been introduced, diagnosed prostate cancer has migrated to earlier stages. In pre-PSA era, only 33% of men diagnosed by DRE had pathologically organ-confined disease. With PSA screening, the percentage of patients with pathologically organ-confined disease is 63–71%.
6) Q: Does PSA screening over-diagnose clinically insignificant[a] prostate cancer?
   A: The vast majority of detected cancers by PSA screening (>80%) appear to be clinically significant, pose a threat to patient life and require definitive treatment.
7) Q: How much earlier can a PSA screening test detect a prostate cancer in comparison to clinical diagnosis?
   A: Three to five years.
8) Q: How many patients with PSA >4 $\mu g\ l^{-1}$ actually have cancer on biopsy?
   A: About 8–15% of men over the age of 50 will have an abnormal PSA test. Of these, most will have benign prostatic hyperplasia or prostatitis. On biopsy, only 20–40% of these patients will have cancer.
9) Q: How many biopsies are necessary to detect one cancer?
   A: On average, out of 4–5 biopsied patients, only one will have prostate cancer.
10) Q: Does PSA screening and early detection extend patient survival?
    A: Initially believed so but there are no data. Prospective studies are underway, and results will be available in about 3–6 years.
11) Q: What is the cost of screening?
    A: In the USA about $10–20 billion per year for all Americans over the age of 50. Estimated cost of $6500 per cancer detected.
12) Q: How can we refine PSA testing for screening?
    A: Measure free/total PSA ratio. Reduces biopsy rate by 20% without compromising sensitivity. Other potential approaches include: Measure PSA density, PSA velocity, use age-adjusted reference ranges.
13) Q: Can prostate cancer be ruled out if total PSA is <4 $\mu g\ l^{-1}$ in men over the age of 50?
    A: No. A small percentage (~5–10%) of patients with PSA between 2.8–4 $\mu g\ l^{-1}$ may have prostate cancer and no suspicious DRE.

[a]These are cancers that are diagnosed at autopsy but are asymptomatic, small and well differentiated, not a threat to the patient's life.

need for bone scans in many patients, thereby saving substantial resources.

Prognostic markers are used to predict the natural history of the disease and the possible response to a specific type of treatment. The best prognostic markers should provide statistically significant information independently from other variables. Pre-operative PSA levels are fairly predictive of patient outcomes (disease-free and overall survival) because they correlate roughly with tumor volume. Pre-operative PSA appears to be an independent prognostic factor before initiation of radiotherapy. Low pre-operative PSA levels strongly predict freedom from biochemical relapse after surgery or radiotherapy. Groups of patients with PSA values between 0–4, 4–10 and 10–20 and >20 $\mu g\ l^{-1}$ have different outcomes after radiotherapy or surgery.

- **PSA in Monitoring Response to Treatment**

After the diagnosis of prostate cancer is made, the question arises as to what is the best treatment for each patient. If there is an indication that the cancer is localized within the prostate and that the patient has a life expectancy greater than ten years, radical prostatectomy is the treatment of choice. Many patients are cured of prostate cancer by this treatment and the frequency of performing this procedure has increased significantly to ~40% of all prostate cancers, partially owing to PSA screening, which usually discovers localized disease. A subset of patients who undergo radical prostatectomy are not cured because of failure to remove the entire tumor mass (those patients are thought to have residual disease after surgery). Other patients are apparently cured, but they relapse at varying intervals after surgery and they might require additional treatment. PSA testing

in post-prostatectomy patients is of paramount importance in deciding who has residual disease, who has relapsed (and when), and who can be considered cured. This has been the most widely accepted application of PSA testing in clinical practice.

Serum PSA in males originates from the prostate gland, with other tissues contributing negligible amounts. Recently, using ultrasensitive PSA assays, we have shown that after radical prostatectomy, serum PSA levels should drop to levels found in women. Indeed, in the majority of apparently cured patients, PSA levels drop to <0.01 μg l^{-1} post-prostatectomy (Yu *et al.* 1997). Because most PSA assays in current use can detect PSA at or above 0.1 μg l^{-1}, the current clinical decision as to who has residual disease can be made about six to eight weeks after radical prostatectomy using a serum PSA measurement. Those whose PSA level drops to <0.1 μg l^{-1} are considered to be in remission; those with a PSA level ≥ 0.1 μg l^{-1} are considered to have residual disease. The latter group of patients is usually associated with positive surgical margins, seminal vesicle involvement, capsular penetration, positive lymph nodes, a Gleason score ≥ 7 and high pre-operative PSA levels. Patients with residual disease usually increase their PSA level continuously after surgery, unless other forms of treatment are instituted (such as radiotherapy or androgen-ablation therapy using pharmacological agents).

The best indicator of post-prostatectomy long-term success is the PSA level, measured at regular time intervals (such as every six months). With conventional PSA testing, a value of <0.1 μg l^{-1} indicates remission. If the PSA starts to rise consistently over time, the patient is considered to have biochemical relapse. This usually precedes any clinical symptoms by about 6–18 months. When the PSA rises to 1–2 μg l^{-1}, the patient is a candidate for additional treatment, usually local radiotherapy or androgen ablation.

With the introduction of ultrasensitive PSA assays, which can measure PSA at 0.01 μg l^{-1} or lower, the current criteria for monitoring are beginning to change. In most patients, post-prostatectomy PSA levels drop below 0.01 μg l^{-1}, and monitoring these patients with the new ultrasensitive assays can reveal biochemical relapse much earlier than when using conventional assays (Prestigiacomo and Stamey 1994, Ferguson *et al.* 1996, Ellis *et al.* 1997, Witherspoon and Lapeyrolerie 1997, Yu *et al.* 1997). An approximate additional lead time of 6–18 months can be gained by using more sensitive PSA assays. The question as to whether the earliest possible intervention would bring the best possible outcome of post-prostatectomy-failed patients, in terms of patient survival and quality of life, needs to be answered with prospective studies. Indications that early intervention does help have been published recently (Diamandis 1997).

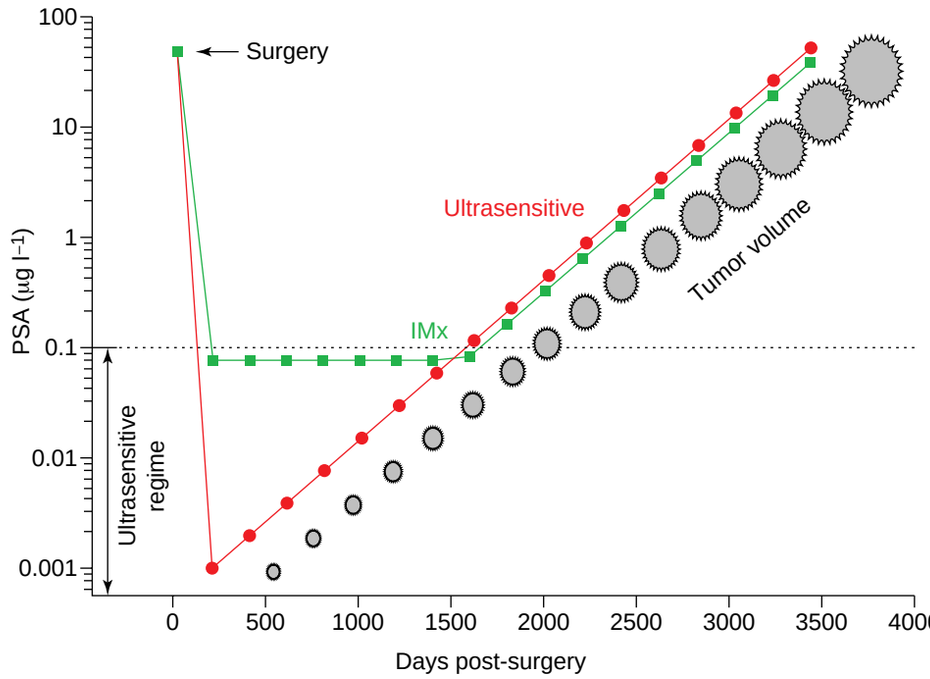

Figure 1. Hypothetical example of a prostate cancer patient who underwent radical prostatectomy and his post-operative prostate-specific antigen (PSA) level was monitored with a commercial assay (Abbott IMx) and an ultrasensitive PSA assay (red line). Notice the earlier detection of relapse with the ultrasensitive assay, at a stage when tumor burden is relatively small. It is assumed that PSA increases exponentially with time.

Figure 1 represents a hypothetical example of post-prostatectomy monitoring with a conventional as well as an ultrasensitive PSA assay. The premise that the more sensitive assays would help patient outcomes is based on the following assumptions:

(1) There is a subgroup of patients whose PSA level does not rise above 0.01 μg l^{-1} during five years of follow-up. Most, if not all, of these patients can be considered cured.

(2) In patients who relapse and their PSA levels are still ~0.1 μg l^{-1}, the tumor might be small, possibly localized and/or more sensitive to treatment. Early treatment of such tumors might produce better results with lower doses, thus avoiding some of the side effects of the treatment.

(3) Not all patients with prostate cancer have tumors with the same aggressiveness. Unfortunately, markers of aggressiveness for prostate cancer are not very accurate at present. Along with other workers, we have postulated that PSA doubling time after surgery might provide information on tumor aggressiveness (Ellis *et al.* 1997, Patel *et al.* 1997, Pruthi *et al.* 1997, Yu *et al.* 1997). An illustrative example is shown in Fig. 2. Calculation of doubling time early after prostatectomy with ultrasensitive assays might aid in patient subclassification based on tumor proliferative potential and aggressiveness.

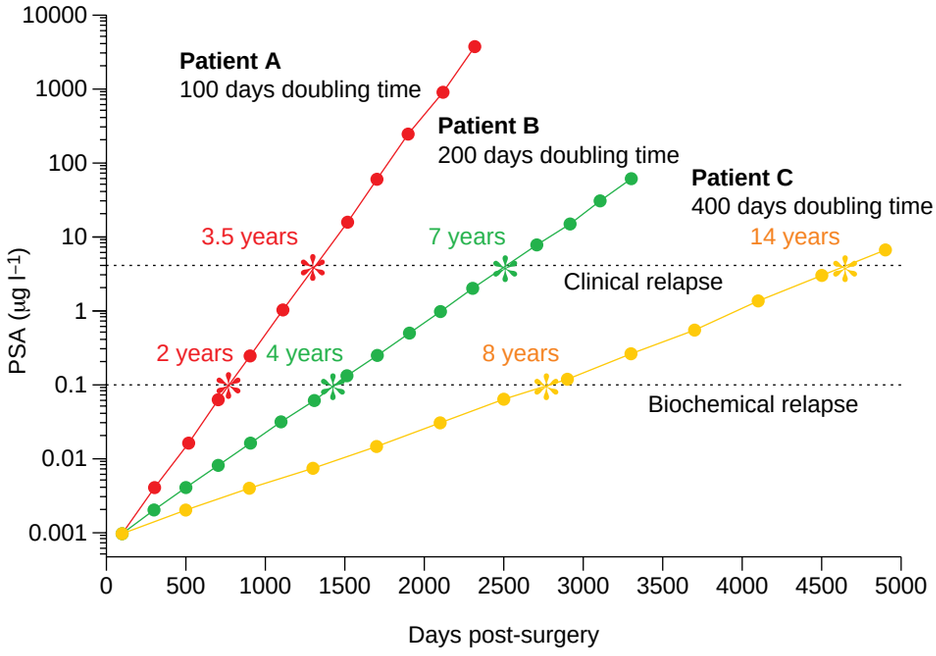

Figure 2. Hypothetical example of the exponential increase in levels of prostate-specific antigen (PSA) with time in three patients who relapsed after radical prostatectomy. In these patients, PSA doubling time was 100 days (**A**), 200 days (**B**) and 400 days (**C**). Notice the difference in time for biochemical relapse (PSA ~0.1 μg l^{-1}) and clinical relapse (PSA ~4 μg l^{-1}).

Post-irradiation PSA monitoring has also been shown to be useful, but the situation is more complicated as the decline in PSA levels after irradiation is not as dramatic as with radical prostatectomy. A PSA nadir around 0.5–1 μg l^{-1} after irradiation is considered a favorable prognostic indicator (Cox *et al.* 1997). Also, the later the elevation of PSA after surgery, the better the chance that these patients will respond to radiotherapy (Cadeddu *et al.* 1998).

Identification of Prostate Cells by Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Cells that can synthesize and secrete PSA contain PSA mRNA. This species can be detected with extremely high sensitivity with RT-PCR. If we accept that PSA is only expressed in prostate cells, then the monitoring of PSA mRNA becomes a powerful tool for detecting prostate cells in various environments. Originally, RT-PCR was used to detect PSA mRNA-containing cells in lymph nodes of patients with prostate cancer. Indeed, this approach is more sensitive than histology and immunohistochemistry for detecting lymph node metastasis (Deguchi *et al.* 1993 and 1997, Diamandis and Yu 1995b), and might be useful for prognosis and selection of treatment in these patients. An extension of these studies was the attempt to detect circulating prostate cancer cells for the purpose of 'molecular staging' of prostate cancer (Corey *et al.* 1997b). Indeed, patients with late-stage disease and distant metastasis are more frequently positive for circulating prostate cancer cells than patients with early-stage disease. The major problem with this technique is that when the sensitivity is adjusted to be high (for example, by using nested primer RT-PCR), non-prostate cancer patients, benign prostatic hyperplasia patients and/or even women can be found to be positive. However, when the sensitivity of the technique is adjusted to be low, many patients with disseminated disease are negative. Recently, other prostatic cell mRNA species were used for the same purpose, including prostate-specific membrane antigen (PSMA) and human kallikrein 2 (hK2) mRNA (Gregorakis *et al.* 1998, Rittenhouse *et al.* 1998). None of these markers at present is established for routine applications.

Table 2. Human kallikrein 3 (PSA): new knowledge and applications[a]

Finding
• Discovery of PSA in breast tumors, normal breast tissue and hyperplastic breast tissue.
• Production of PSA by breast cancer cell lines, induced by androgens and progestins.
• Presence of PSA in milk of lactating women and breast cyst fluid.
• Presence of PSA in amniotic fluid; possible association with fetal abnormalities.
• PSA presence in various tumors.
• PSA in serum of women/possible use for disease diagnosis with PSA subfractions.
• PSA as a prognostic indicator in breast cancer.
• PSA in ovarian/lung cancer.
• PSA in nipple aspirate fluid; breast cancer risk assessment.
• Serum PSA as a marker of androgen excess in women.
• Mutations of the promoter region of the PSA gene in breast cancer.

[a]Reprint from Diamandis (1998). Specific references are included in that article.

- **Non-prostatic PSA**

Initially, the workers who discovered the PSA protein tested many human tissues for PSA expression, but none of them was positive. The term prostate-specific antigen was fully justified until a few years ago when PSA was identified at relatively low levels in diverse tissues, but predominantly in female breast tissue and its secretions (Diamandis and Yu 1995a, Borchert *et al.* 1997). The expression of PSA in breast tissue is regulated by steroid hormones. In addition to clinical studies suggesting a prognostic value for

PSA expression in breast tumors (Yu *et al.* 1995), the potential for breast cancer risk assessment by measuring nipple aspirate fluid PSA (Sauter *et al.* 1996) and diagnostic applications based on serum PSA measurements in women (Melegos and Diamandis 1996), there are tissue culture systems based on breast carcinoma cell lines that are valuable for studying PSA regulation (Zarghami *et al.* 1997). Because PSA is a protease, there are suggestions that PSA might have a biological function in the breast that is connected with this activity, such as the activation of latent growth factors (or cytokines), degradation of binding proteins for growth factors, or intrinsic growth regulating activity (Diamandis and Yu 1995, Peehl 1995). These possibilities are now under investigation. Table 2 summarizes the extra-prostatic sources of PSA (Diamandis 1998).

- **Conclusion**

PSA is the best tumor marker currently available. Its clinical value for prostate cancer screening, diagnosis, prognosis and monitoring is well established. More recently, clinical applications of PSA are starting to extend beyond the prostate, most prominently in breast cancer. These two cancers have many epidemiological, molecular and biochemical similarities (Lopez-Otin and Diamandis 1998). Currently, there are efforts to supplement PSA diagnostics with other markers including PSMA (Gregorakis *et al.* 1998) and hK2 (Rittenhouse *et al.* 1998). Others are trying to take advantage of the unique expression of PSA in prostate tissue to deliver gene therapy (Cleutjens *et al.* 1997a). This field of investigation continues to grow rapidly.

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TEM changes in style of citations to go into effect as of 1/1/99 (in order to match the style of other Trends journals published in Cambridge)

- References are cited in the text by consecutive numbers in superscript. When two are cited in order the format is[11,12]; where more than two[11–14]. The only exception to this rule is where the reference immediately follows an abbreviation, acronym or chemical name, at which point the references should be cited as (Refs 11, 12). References in reviews are quoted as (reviewed in Ref. 13).
- If unpublished material is cited, it is referred to as (M.K. Smith and B. Jones, unpublished) or (R. Brines, pers. commun.). Keep such citations to a minimum, and avoid them for data important to the gist of the review. Numbered references must be at least in press.
- A reference list of not more than 40 references should be provided.
- Not alphabetical, but in numerical order (same order as in text). Introduce each with the appropriate number (**1** to **54** etc.) in bold Roman typeface.
- One author: Blair, T.W. (1994). **Title** (bold Roman). *Endocrinology* 125, 334–337
- Two authors: Blair, T.W. and Thatcher, M. (1996). **Title**. *J. Endocrinol.* 39, 45–51
- Up to six authors: Blair, T.W., Thatcher, M., Carroll, H. and Woo, P.T.K. (1993). **Title**. *J. Biol. Chem.* 239, 23356–23382
- More than six authors: Blair, T.W. *et al.* (1998). **Title**. *Proc. Natl Acad. Sci. USA* 101, 1111-1121
- Chapter in book: Woo, P.T.K. and Wehnert, S.D. (1993). **Title**. in *Parasitic Protozoa* (Vol. 2) (2nd edn) (Kreier, J.F. and Baker, J.R., eds) pp 157–256, Academic Press
- Abstract: instead of last page, write (abstr).
- Common abbreviations: *Eur. J. Immunol.*
  Proc. Natl Acad. Sci. USA
  J. Exp. Med.
- One word titles are spelt out in full e.g. *Nature, Cell, Science* etc.
- Note: several issues ago, TEM switched from mg/mL to mg ml^{-1} (in all similar expressions).
- Cite figures as (Fig. 1 or Fig. 2a).