Applicability of biomarkers in the early diagnosis of prostate cancer

Daphne Hessels, Gerald W Verhaegh, Jack A Schalken and J Alfred Witjes[†]

Early diagnosis of prostate cancer can increase the curative success rate for this disease. Although serum prostate-specific antigen measurement is regarded as the best conventional tumor marker available, there is little doubt that it has great limitations. The threshold above which biopsies are indicated has now decreased to a serum prostate-specific antigen value of 3 ng/ml, which results in a negative biopsy rate of 70–80%. This can readily be explained by the fact that prostate-specific antigen is not specific for prostate cancer. Clinicians need more sensitive tools to help diagnose prostate cancer and monitor progression of the disease. Molecular oncology is playing an increasing role in the fields of diagnosis and therapy for prostate cancer and has already been instrumental in elucidating many of the basic mechanisms underlying the development and progression of this disease. The identification of new prostate cancer-specific genes, such as *DD3*[PCA3], would represent a considerable advance in the improvement of diagnostic tests for prostate cancer. This could subsequently lead to a reduction of the number of unnecessary biopsies.

CONTENTS

[†]*Author for correspondence Department of Urology, University Medical Center Nijmegen, 6525 GA Nijmegen, The Netherlands Tel.: +31 24 361 6712 Fax: +31 24 354 1031 F.Witjes@uro.umcn.nl*

KEYWORDS:
body fluids, diagnosis, diagnostic tests, epigenetic modifications, gene expression, human kallikreins, molecular techniques, molecular tumor markers, prostate cancer, serum markers

In the Western male population, prostate cancer has become a major public health problem. In many developed countries, it is not only the most commonly diagnosed malignancy but also the second leading cause of cancer-related deaths. Since the incidence of prostate cancer increases with age, the number of newly diagnosed cases continues to rise as the life expectancy of the general population increases. Approximately 230,110 men in the USA and 85,000 men in Europe are newly diagnosed with prostate cancer each year [1,2]. Epidemiology studies indicate that prostate cancer is an indolent disease and that more men die with prostate cancer than from it. However, 25–30% of the tumors behave aggressively and as a result, around 29,900 US and 35,000 European men die from this disease annually. The high mortality rate is a consequence of the fact that there are no adequate therapeutic options for metastatic prostate cancer. Androgen ablation is the treatment of choice in men with metastatic disease. Initially, 70–80% of patients with advanced disease show a response to therapy. However, within 2–3 years, the majority of tumors become androgen independent and more aggressive. As a result, most patients will develop progressive disease. Currently, there is no effective treatment for this hormone-refractory stage of the disease. More than 70% of hormone-refractory patients suffer from painful bone metastases, which are the most important cause of morbidity.

Prostate cancer detection

Radical prostatectomy and radiotherapy are curative therapeutic options for prostate cancer but are limited to organ-confined disease. Early detection of prostate cancer, when the disease is confined to the prostate, is therefore pivotal. Since its discovery more than 20 years ago, prostate-specific antigen (PSA) has been the most valuable tool in the detection, staging and monitoring of prostate cancer. The European Randomised Study of Screening for Prostate Cancer (ERSPC), Rotterdam section, continuously evaluates screening procedures. Based on the knowledge of tumor characteristics and prevalence predictions of biopsy-detectable cancers per

PSA range, this ERSPC study group implemented changes in the screening protocol. Since 1997, the ERSPC study group accepted PSA values of more than or equal to 3 ng/ml as the standard biopsy indication. Digital rectal examination (DRE) and transrectal ultrasound (TRUS) have been entirely discarded as initial screening tests [3,4]. A large multicenter prostate cancer screening trial showed that approximately 50% of patients with serum PSA values greater than 10 ng/ml had advanced disease. Most patients with serum PSA values less than 10 ng/ml were diagnosed with early stage disease [5]. These findings have led to the conclusion that men with serum PSA values between 3 and 10 ng/ml most likely have clinically localized disease and would benefit from curative treatment. Due to the growing awareness of prostate cancer in the Western population and the introduction of the serum PSA test, the numbers of men newly diagnosed with localized prostate cancer has increased. However, concerns have arisen regarding the detection of clinically insignificant prostate cancers. The latter are tumors that do not pose a serious life threat and, as a result, do not require therapy. The tantalizing question for clinicians and researchers is how to distinguish a potentially dangerous from an indolent tumor.

Although widely accepted as a prostate tumor marker, PSA has turned out to be organ specific but not prostate cancer specific. PSA levels have been reported to be increased in men with benign prostatic hyperplasia (BPH) and prostatitis. This substantial overlap in serum PSA values between men with nonmalignant prostatic diseases and prostate cancer is the limitation of PSA as a prostate tumor marker. Moreover, PSA cannot be used to differentiate the aggressive from the indolent tumors. Upon detection of serum PSA values greater than 3 ng/ml, the conventional diagnostic approach is traditional sextant TRUS-guided prostate biopsies. However, the low specificity of serum PSA results in a negative biopsy rate of 70–80%. In some cases, biopsy specimens may not be representative, which also attributes to the failure to detect some cancers. Currently, most academic centers recommend extension of the diagnostic set to ten biopsies. In case of persistent rising serum PSA levels, repeated biopsies are indicated, which have at least 10% probability of finding cancer [6]. Moreover, if the combined use of serum PSA, DRE and TRUS biopsy do indicate clinically confined cancer, 40% of these men are found to have already extracapsular disease upon radical prostatectomy [7]. Therefore, noninvasive screening tests that can accurately identify men who have early stage, organ-confined prostate cancer and who would gain prolonged survival and improved quality of life from early radical intervention are urgently needed.

New prostate cancer screening tests

New prostate cancer screening tests have to meet four basic requirements to be an effective and practical approach for early detection [8]:

- Accurately discriminate between healthy males or males with nonmalignant prostatic diseases and males with prostate cancer, with both high sensitivity and high specificity

- Detection of prostate cancer should occur when the cancer is still confined to the prostate and radical treatment is potentially curative

- Distinguish the indolent from the aggressive tumors to avoid the problem of over diagnosis

- Be well accepted by the population targeted for screening and be inexpensive

As can be concluded from above, PSA as a screening marker for prostate cancer does not fulfil these requirements. The problem lies in the fact that *PSA* is not a prostate cancer-specific gene and does not discriminate between indolent and aggressive tumors. One approach to improve diagnostic accuracy of tests for prostate cancer and reduce the number of unnecessary biopsies is through the identification of prostate cancer-specific genes.

For the identification of new candidate markers for prostate cancer, it is necessary to study expression patterns in malignant as well as nonmalignant prostate tissues. Recent developments in molecular techniques have provided new tools that enable the comprehensive and rapid assessment of both genomic and proteomic alterations in samples. For instance, the identification of different chromosomal abnormalities such as changes in chromosome number, translocations, deletions, rearrangements and duplications in cells can be studied using fluorescence *in situ* hybridization (FISH) analysis. Comparative genomic hybridization (CGH) is able to screen the entire genome for large changes in DNA sequence copy number or deletions larger than 10 Mbp. Differential display analysis, serial analysis of gene expression (SAGE), oligonucleotide arrays and complementary DNA (cDNA) arrays characterize gene expression profiles. These techniques are often used in combination with tissue microarrays (TMAs) for the identification of genes that play an important role in specific biological processes [9].

Since genetic alterations often lead to mutated or altered proteins, the signaling pathways of a cell may become affected. Eventually, this may lead to a growth advantage or survival of a cancer cell. Proteomics is the term that refers to the identification of altered proteins in terms of structure, quantity and posttranslational modifications. Disease-related proteins can be directly sequenced and identified in intact whole tissue sections using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS). Additionally, surface-enhanced laser desorption/ionization (SELDI)-TOF MS can provide a rapid protein expression profile from tissue cells and body fluids such as serum or urine [10].

In the last 5 years, these molecular tools have led to the identification of hundreds of genes that are believed to be relevant in the development of prostate cancer. Not only have these findings led to a greater insight into the initiation and progression of prostate cancer, they have also shown that prostate cancer is a very heterogeneous disease. Several prostate tumors may occur in the prostate of a single patient due to the multifocal nature of the disease. Each of these tumors can show remarkable differences in gene expression and behavior that are associated

with varying prognoses. Therefore, in predicting the outcome of the disease, it is more likely that a set of different markers will become clinically important.

In 2003, an overview was given on prostate cancer biomarkers for cancer diagnosis, prognosis and prediction of disease survival [11]. This review will focus on the available data concerning the applicability of both old and new biomarkers in the early detection of prostate cancer in body fluids, such as urine and serum. These biomarkers are classified into four different prostate cancer-specific events: genomic alterations, prostate cancer-specific biological processes, epigenetic modifications and genes uniquely expressed in prostate cancer. Furthermore, this review will focus on whether current tests developed for several markers have the potential to reduce the number of unnecessary biopsies in men with total serum PSA values over 3 ng/ml.

Genomic alterations in prostate cancer
Prostate cancer-associated gene mutations
One of the strongest epidemiological risk factors for prostate cancer is a positive family history. A study of 44,788 pairs of twins in Denmark, Sweden and Finland has shown that 42% of prostate cancer cases were attributable to inheritance [12]. It has been observed that brothers of affected patients are consistently at higher risk for the disease compared with the sons of the same patients. This has led to the hypothesis that there is an X-linked or recessive genetic component involved in the risk for prostate cancer [13]. Genome-wide scans in affected families implicated at least seven prostate cancer-susceptibility loci: *HPC1* (1q24), *CAPB* (1p36), *PCAP* (1q42), *ELAC2* (17p11), *HPC20* (20q13), 8p22–23 and *HPCX* (Xq27–28). Recently, three candidate hereditary prostate cancer genes have been mapped to these loci: HPC1/2´-5´-oligoadenylate dependent ribonuclease L (*RNASEL*) on chromosome 1q24–25, macrophage scavenger 1 gene (*MSR1*) located on chromosome 8p22–23 and *HPC2/ELAC2* on chromosome 17p11 [14].

It has been estimated that prostate cancer susceptibility genes account for only 10% of prostate cancer cases. Familial prostate cancers are most likely associated with shared environmental factors or more common genetic variants of polymorphisms. Since such variants may occur at high frequencies in the affected population, their impact on prostate cancer risk can be substantial. Recently, polymorphisms in the genes coding for the androgen-receptor (AR), 5α-reductase Type II (SRD5A2), CYP17, CYP3A, vitamin D receptor, PSA, GST-T1, GST-M1, GST-P1, insulin-like growth factor (IGF)-I and IGF-binding protein-3 have been studied. These studies were performed to establish whether these genes can predict the presence of prostate cancer in patients indicated for prostate biopsies due to PSA levels greater than 3 ng/ml. No associations were found between the AR, SRD5A2, CYP17, CYP3A4, vitamin D receptor, GST-M1, GST-P1 and IGF-binding protein-3 genotypes and prostate cancer risk. Only GST-T1 and IGF-I polymorphisms were found to be modestly associated with prostate cancer risk [15].

Unlike the adenomatous polyposis coli (APC) gene in familial colon cancer, none of the mentioned prostate cancer susceptibility genes and loci were by themselves responsible for the largest portion of prostate cancers. Epidemiology studies support the idea that most prostate cancers can be attributed to factors such as race, lifestyle and diet. The role of gene mutations in known oncogenes and tumor suppressor genes is probably very small in primary prostate cancer. For instance, the frequency of p53 mutations in primary prostate cancer is reported to be low but has been observed in almost 50% of advanced prostate cancers [16,17].

Screening men for the presence of cancer-specific gene mutations or polymorphisms is time consuming and costly. Moreover, it is very ineffective in the detection of primary prostate cancers in the general male population. Therefore, it cannot be applied as a prostate cancer screening test.

Mitochondrial DNA alterations
Mitochondrial DNA is present in approximately 1000 to 10,000 copies per cell [18]. Due to these quantities, mitochondrial DNA mutations have been used as a target for the analysis of plasma and serum DNA from prostate cancer patients. Recently, mitochondrial DNA mutations were detected in all three prostate cancer patients who had the same mitochondrial DNA mutations in their primary tumor [19]. Different urological tumor specimens must be studied and larger patient groups are needed to define the overall diagnostic sensitivity of this method.

Microsatellite alterations
Critical alterations in gene expression can lead to the progression of prostate cancer. Microsatellite alterations, which are polymorphic repetitive DNA sequences, often appear as loss of heterozygosity (LOH) or as microsatellite instability. Defined microsatellite alterations are known in prostate cancer. However, for the detection of microsatellite instability, the ratio of tumor to normal must be greater than 0.5%. For the detection of LOH, at least 20% of the analyzed genomic DNA must be obtained from tumor cells [20]. This might become a problem in blood or urine samples, in which a single prostate cancer cell must be detected in a huge background of normal cells. Furthermore, microsatellite analysis using small amounts of DNA is prone to artefacts. Due to the low amounts of genomic DNA, microsatellite analysis and LOH may fail in the detection of tumor DNA in body fluids.

Prostate cancer-specific biological processes
Since many proteins are shed into the circulation as a consequence of disease progression, screening the blood for overexpressed proteins appears to be an excellent way to search for new prostate cancer biomarkers. From recent studies, there is growing evidence that kallikreins and kallikrein-like genes are related to many types of malignancies. Recently, it has been suggested that there may be crosstalk between the kallikreins and that they participate in pathways that affect normal

physiological or pathological processes [21]. PSA is a member of the kallikrein family and since it has been successful in the diagnosis of prostate cancer, it is assumed that other kallikreins may also have diagnostic potential in prostate cancer.

Recently, 15 members of this gene family have been identified on chromosome 19q13.3–13.4. These 15 genes have a highly conserved structural organization and encode for putative secreted proteases. Their enzymatic activity may initiate or terminate biological events such as angiogenesis and growth factor release [22]. The release of these proteases into the blood circulation may reflect growth patterns of prostate cancer.

PSA & serum subforms of PSA

PSA, encoded by the *KLK3* gene, is the most studied biomarker. Its drawbacks in the screening for prostate cancer have already been mentioned in this review. PSA is not overexpressed in prostate cancer cells. In fact, the PSA messenger RNA (mRNA) expression is approximately 1.5-fold lower in prostate tumor tissue compared with normal prostate tissue [23]. Therefore, it is assumed that the increase in serum PSA expression is a result of cancer progression. In the normal prostate, most of the produced PSA will be excreted into the semen where it acts as an androgen-regulated serine protease. Only a small amount of PSA will leak into the blood circulation. It is speculated that due to tumor development, the tissue architecture is altered by the disruption of the basal cell layer and basement membrane. It has been demonstrated that prostate cancer tissue releases 30-times more PSA into the circulation than normal prostate tissue [24]. Circulating PSA can occur in the serum either in an unbound free form or it can be bound to α1-antichymotrypsin or α2-macroglobulin.

There have been several attempts to improve the specificity of total PSA for the detection of prostate cancer in the range of 3 to 10 ng/ml, the so-called diagnostic PSA gray zone. These improvements include PSA density, transition zone PSA density, PSA velocity and age-specific PSA. These methods, all based on calculations with total PSA, have not been effective in the diagnostic gray zone to reduce the number of unnecessary biopsies [25,26].

Percent free PSA

Studies on percent free PSA (%fPSA), which is the ratio of free PSA to total PSA, appeared promising to increase the specificity in the diagnostic gray zone. Significantly higher %fPSA values have been observed in the serum of patients with BPH compared with those found in the serum of patients with prostate carcinomas. As a consequence, the probability of prostate cancer increased with a decrease of %fPSA values [27]. In patients with total serum PSA values of 4–10 ng/ml, %fPSA values at or below 25% resulted in the detection of 95% of prostate cancers upon biopsy and a reduction of 20% of unnecessary biopsies. Using a %fPSA cut-off value of 22% in the same group of patients, the sensitivity dropped to 90% but led to a 29% reduction of unnecessary biopsies [28].

On the other hand, patients with chronic prostatitis were demonstrated to have %fPSA values that are comparable with those of patients with prostate cancer [29]. This might lead to false-positive indications and thus to unnecessary biopsies. To date, controversy exists over using %fPSA values in addition to total PSA in screening for prostate cancer [30–32]. However, it has been reported that %fPSA appears to be more effective in the decision making of a repeat biopsy following an initial negative biopsy. In this way, a %fPSA cut-off value of 30% is optimal, leading to 90% sensitivity and a reduction of 50% of unnecessary biopsies [6].

ProPSA

Free PSA in serum is composed of distinct forms of inactive PSA, as reviewed by Mikolajczyk and coworkers [33,34]. ProPSA, which is one of the free PSA compounds in serum, is the name for native proPSA ([-7]proPSA) as well as truncated proPSA forms ([-2]proPSA, [-4]proPSA and [-5]proPSA). It has been demonstrated that in the diagnostic gray zone, total PSA had a specificity of 23%, %fPSA had a specificity of 33% and proPSA had a specificity of 13% at 90% sensitivity. A combination of these three variables resulted in a specificity of 44% in the diagnostic gray zone, which remarkably improved the specificity for early prostate cancer detection [35].

In a recent study, the development of highly sensitive and specific immunoassays for all intact and truncated forms of proPSA was described [36]. This study showed that the percentage of all proPSA forms (sum-proPSA/fPSA; area under the curve [AUC] of 0.69) is able to improve the detection of prostate cancer compared with %fPSA (AUC of 0.63) and total PSA (AUC of 0.53) in the PSA gray zone used here of 4–10 ng/ml. Overall, %[-2]proPSA ([-2]proPSA/fPSA) showed the same diagnostic potential as %sum-proPSA. However, in men with high (>25%) %fPSA values, %[-2]proPSA showed better diagnostic potential than %sum-proPSA. In these samples, %[-2]proPSA would have spared 36% of men from unnecessary biopsies, whereas %sum-proPSA would only have spared 29% of men. The additional forms of PSA mentioned here expand the possibilities for the use of PSA in the detection of prostate cancer.

hK2

There have been many studies on hK2, encoded by the *KLK2* gene, which is another member of the kallikrein family. Like PSA, hK2 expression is regulated by androgens and has been detected in several other biological fluids, such as amniotic fluid, breast milk, breast cyst fluid and in malignant and non-malignant breast tissues. *In vitro* studies have shown that active hK2, prostase and hK15, which are also expressed at high levels in the prostate, convert latent proPSA into active PSA. The genes encoding hK2 and PSA show 80% homology and the two proteins show 78% amino acid sequence identity. hK2 is found at much lower concentrations (1–2% of PSA concentrations) in prostate tissue. The presence of hK2 in seminal plasma, albeit at only 0.1–1% of PSA concentrations,

suggests that hK2 is also involved the cleavage of gel-forming proteins. The concentration of hK2 in serum is less than 3% of the PSA concentrations and, unlike PSA, it exists mainly in the free, unbound form. The development of specific hK2 immunoassays has been hampered by the high homology with PSA and the very low hK2 concentrations. This has resulted in highly sensitive assays for hK2 with very low detection limits (<10 ng/l) [37].

Like PSA, hK2 was expressed less in malignant prostate tissue compared with nonmalignant prostate tissue, albeit that the downregulation was less for hK2 than for PSA [38]. In a study where gene expression of PSA and KLK2 in Gleason grade (G) 4 or 5 tumors were compared with the gene expression of both genes in BPH specimens, both genes were found to be more than tenfold overexpressed compared with other genes [39]. However, there was no difference in gene expression between G4/5 tumors and the BPH samples. The clinical use of hK2 as a replacement for total PSA based on relative gene expression levels did not appear to be promising. Recently, it was shown that serum hK2 values alone were no more discriminatory than total PSA (TABLE 1) [40].

However, it has been demonstrated that the ratio of serum hK2 to free PSA could better distinguish prostate cancer from BPH than total PSA. In the diagnostic gray zone at 100% sensitivity, the ratio of hK2 to free PSA had a specificity of 48.2% [41]. This would have spared unnecessary biopsies in half of the men with elevated PSA levels.

Recently, it was shown that total PSA, free PSA or PSA-α1-ACT were not able to distinguish the poorly differentiated G3 tumors from the moderately differentiated G1 and G2 prostate tumors [42]. However, hK2 significantly improved the identification of G3 prostate tumors compared with %fPSA ratio. Furthermore, multivariate regression analysis revealed that combinations of hK2/free PSA and free PSA/(total PSA × hK2) were significant predictors of G3 tumors in the PSA range of 3 to 15 ng/ml. The free PSA/(total PSA × hK2) ratios were also helpful in prediction of organ-confined disease. Higher ratios showed a better chance for curative treatment and lower ratios showed a lower chance for successful surgery.

Furthermore, it has been determined that serum hK2 levels are remarkably higher in patients with advanced disease (median 116 ng/l) compared with patients with low Gleason scores (median 72 ng/l) or healthy men (median 26 ng/l) [43]. Moreover, serum hK2 levels alone or in combination with total PSA and free PSA have the power to distinguish pathologically organ-confined prostate cancer from locally advanced disease in patients with total PSA values less than 10 ng/ml [44].

These studies show that the ratio of hK2 to free PSA has diagnostic applicability in the diagnostic gray zone to distinguish prostate cancer patients from men with BPH (TABLES 1 & 2). As such, it can lead to a reduction of the number of unnecessary biopsies. Additionally, serum hK2 alone or in combination with total PSA and free PSA may improve the detection of extraprostatic or advanced disease. Multivariate regression analysis including hK2 may become more important for prostate cancer

diagnosis since it allows the more accurate prediction of tumor grade, stage or organ-confined disease.

KLK4

KLK4, which encodes the hK4/prostase protein, is one of the more recently discovered members of the kallikrein gene family. Initial studies using northern blot analysis indicated that KLK4 expression was restricted to the prostate [45]. However, reverse transcription (RT)-PCR analysis demonstrated high KLK4 mRNA expression in prostate, testis, adrenals, uterus and thyroid. KLK4 expression was shown to be regulated either by androgens in the prostate cancer cell line LNCaP, or by androgens and progestins in the breast cancer cell line BT-474 [46]. Using RT-PCR and immunohistochemistry (IHC) experiments, it has been shown that KLK4 is expressed both at the mRNA and protein level in normal human prostate tissues, primary prostate cancer tissues and metastatic prostate cancer tissues [47]. KLK4 mRNA expression was found to be higher in the majority of prostate cancer tissues compared with matched normal prostate tissues [48,49]. RNA *in situ* hybridization studies on normal and hyperplastic prostate tissue specimens in TMAs indicated that KLK4 is predominantly expressed in basal cells of the normal prostate [49].

Surprisingly, the hK4 protein concentrations were found to be frequently lower in the prostate cancer tissues compared with the matched normal prostate tissues. Furthermore, the hK4 protein concentration was found to be unexpectedly low in normal prostatic extracts ($>10^4$-fold lower than PSA) and seminal plasma (10^4–10^6-fold lower than PSA) [48]. Detailed mapping of the KLK4 mRNA 5´-end provided evidence that hK4 does not contain a signal peptide that would target the molecule for secretion. Furthermore, hK4 was found to be predominantly localized in the nucleus using immunofluorescence and cell fractionation experiments [49]. However, KLK4-specific antibodies have been detected in the sera of prostate cancer patients. These antibodies could have been generated by the immune system of prostate cancer patients after recognition of the hK4 protein. It is not known whether the protein enters the blood circulation in individuals with or without prostatic disease [47].

All these data suggest that KLK4 has a unique structure and function compared with the other members of the kallikrein family. In ovarian cancer, KLK4 expression appears to be associated with advanced ovarian carcinomas that have an unfavorable prognostic outcome of disease. Further studies are needed to assess the value of KLK4 mRNA or hK4 protein as biomarkers for prostate cancer.

hK11

The *KLK11* gene encodes hK11, formerly known as hippostasin, trypsin-like serine protease or PRSS20. The protein hK11 was found to be highly expressed in the prostate. A highly sensitive hK11 immunofluorometric assay has been developed and high serum hK11 values were determined in 60% of prostate cancer patients [50]. Recently, it has been demonstrated that the

Table 1. Current biomarker-based tests and their sensitivity and specificity in the diagnosis of prostate cancer in the diagnostic prostate-specific antigen gray zone.

Biomarker	Application	Method	AUC	Sensitivity (%)	Specificity (%)	NPV (%)	Ref.
PSA	Serum	Hybritech tandem PSA assays or dual ProStatus assay	0.50–0.70	90	10–31		[28,35,36,40]
%fPSA	Serum	Hybritech tandem free PSA assays or dual ProStatus assay	0.53–0.76	90	10–45		[28,35,36,40, 41,51]
%sum-proPSA	Serum	Research use dual monoclonal antibody immunoassay	0.69	90	31		[36]
Combination of total PSA, %fPSA and %sum-proPSA	Serum	Research use dual monoclonal antibody immunoassay	0.77	90	44	85	[35]
%[-2]proPSA	Serum	Research use dual monoclonal antibody immunoassay	0.64	90	21		[36]
hK2	Serum	In-house research immunofluorometric assay	0.68	90	20		[40]
fPSA/(tPSAxhK2)	Serum	Immunofluorometric assay for hK2	0.75	88	57	85	[42]
hK2/fPSA	Serum	Immunofluorometric assay for hK2	0.86	100	48.2		[41]
hK11/tPSA	Serum	Immunofluorometric assay for hK11	0.77	90	51.5		[51]
GSTP1	Urine	MSP analysis		73	98	80	[68]
Telomerase	Urine	Telomeric repeat amplification assay		58	100	55	[79]
DD3[PCA3]	Urine	Quantitative RT-PCR assay	0.72	67	83	90	[85]
AMACR	Biopsy tissue	Immunohistochemistry		97	100		[74]

%fPSA: Percent free PSA; AMACR: α-methylacyl-CoA racemase; AUC: Area under curve; GSTP1: Glutathione S-transferase P1; MSP: Methylation-specific PCR; NPV: Negative predictive value; PSA: Prostate-specific antigen; RT: Reverse transcription.

serum hK11 values were significantly lower in prostate cancer patients compared with men with BPH. In this group of men with BPH, 45% would have avoided prostate biopsies based on %fPSA values of over 20%. When the ratio of hK11 to total PSA (>0.05) was applied, another 51.5% of these men with BPH would not have to undergo unnecessary biopsies. At 90% sensitivity, the ratio of hK11 to total PSA (0.05) had a specificity of 51.5% [51]. This indicates that in addition to PSA, %fPSA values combined with hK11 to total PSA ratios can lead to a higher reduction of unnecessary biopsies (TABLE 1). Future studies involving more patients are needed to confirm these preliminary data.

KLK14
KLK14 is another member of the kallikrein gene family. KLK14 expression has been found in the CNS and endocrine-related tissues, such as prostate, thyroid and testis [52]. *In situ* hybridization studies have shown KLK14 mRNA expression in secretory epithelial cells of the normal prostate, prostatic intraepithelial neoplasia (PIN) and malignant prostate cells [53].

This expression pattern is similar to that observed for PSA and hK2. In a recent study, a median 2.15-fold overexpression of KLK14 mRNA has been found in prostate cancer tissues compared with normal prostate tissues. Elevated expression levels of KLK14 appear to correlate with advanced and more aggressive tumors [54]. The potential of KLK14 in the diagnosis of prostate cancer is yet to be determined.

KLK15
The *KLK15* gene has recently been identified. *KLK15* is primarily expressed in the thyroid gland but expression has also been observed in the prostate, salivary gland, adrenal glands, colon, testis and kidney, albeit at much lower levels. Studies in the LNCaP prostate cancer cell line have shown that KLK15 expression was upregulated by steroid hormones. In prostate cancer tissues, KLK15 mRNA was found to be overexpressed compared with normal prostate tissues [55]. A recent study using quantitative real-time PCR has shown an association between the upregulation of KLK15 and advanced and more aggressive prostate tumors [56]. With KLK15 being a member of the family

of serine proteases, these high mRNA levels may indicate the presence of a protein. When shed into the serum as a result of cancer development, serum KLK15 may become important as a diagnostic and prognostic marker for prostate cancer.

50.8-kDa protein

Using peptide mass fingerprinting, a 50.8-kDa protein (previously known as NMP48) was shown to be related to a vitamin D-binding protein. The protein has been detected in the sera of 96% of prostate cancer-affected men and in 53% of men with high-grade PIN. No expression has been found in the sera of men with benign prostates (75%), BPH (70%), status after radical prostatectomy (80%) or healthy controls (96%). This small preliminary study indicates a role for this protein as a biomarker for the early detection of prostate cancer (TABLE 2) [57].

These preliminary studies indicate that a combination of these serum markers can remarkably improve the specificity for the detection of prostate cancer (TABLE 1). In particular, the combination of serum hK2 or hK11 with different forms of serum PSA has the potential to reduce the number of unnecessary biopsies in the diagnostic gray zone. Moreover, serum hK2 may improve the detection of extraprostatic disease and KLK15 may be used to discriminate between the more aggressive and indolent tumors.

Support for using a panel of proteins came from a recent study in which a novel technique, similar to SELDI-TOF MS, was combined with bioinformatics. It showed that complex serum protein profiles have the potential to identify prostate cancer and that the combination of two array types with different surface chemistries increased the number of more clinically significant discriminators. This new tool had an 85% sensitivity and specificity for the detection of prostate cancer [58]. In addition, such a serum-based proteomic pattern analysis has already proven to be successful in the early detection of ovarian cancer [59]. Using this method, the diagnosis of ovarian cancer was based on a panel of individual proteins, each of which was not independently discriminatory for the disease.

Epigenetic modifications

Alterations in DNA, without changing the order of bases in the sequence, often leads to changes in gene expression. These epigenetic modifications include changes such as DNA methylation and histone acetylation/deacetylation. Many gene promoters contain GC-rich regions known as CpG islands. Abnormal methylation of CpG islands results in decreased transcription of the gene into mRNA. Recently, it has been suggested that the DNA methylation status may be influenced in early life by environmental exposures, such as nutritional factors or stress, and that this leads to an increased risk for cancer in adults [60]. Changes in DNA methylation patterns have been observed in many human tumors [61]. A technique known as methylation-specific PCR (MSP) is used for the detection of promoter hypermethylation. In contrast to microsatellite or LOH analysis, this technique requires a tumor-to-normal ratio of only 0.1–0.001%. This means that by using this technique, hypermethylated alleles from tumor DNA can be detected in the presence of 10^4–10^5 excess amounts of normal alleles [62]. Therefore, DNA methylation

Table 2. Biomarkers and their potential application in the detection of prostate cancer.

Goal	Biomarker	Application	Method	Ref.
Discrimination of organ-confined disease from locally advanced disease	hK2, fPSA/(tPSAxhK2)	Serum	Immunofluorometric assays	[42–44]
Prediction of grade 3 tumors	hK2/fPSA, fPSA/(tPSAxhK2)	Serum	Immunofluorometric assays	[42]
Distinguish the more aggressive tumors from the indolent ones	Hepsin	Serum	Immunofluorometric assays	[77]
	RASSF1A	Urine	MSP analysis	[63,64]
Reduction of the number of unnecessary biopsies	50.3-kDa protein, PSMA, hK11/tPSA, hK2/fPSA, combination of tPSA, %fPSA and %sum-pro-PSA	Serum	Immunofluorometric assays	[57,83] [41,51] [35]
	GSTP1	Urine	MSP analysis	[68]
	DD3^{PCA3}	Urine	Quantitative RT-PCR	[85]
Candidate for molecular probe in imaging modalities	AMACR		Imaging modalities	[73]
Prediction of disease progression (circulating tumor cells)	PSMA/PSMA	Tissue, blood	Quantitative RT-PCR	[81]
	DD3^{PCA3}	Blood		[86]

%fPSA: Percent free PSA; AMACR: α-methylacyl-CoA racemase; GSTP1: Glutathione S-transferase P1; MSP: Methylation-specific PCR; PSA: Prostate-specific antigen; PSMA: Prostate-specific membrane antigen; RASSF1A: Ras-association domain family protein isoform A; RT: Reverse transcription.

can serve as a useful marker in cancer detection. Recently, there have been many reports on hypermethylated genes in human prostate cancer. Two of these genes are Ras-association domain family protein isoform A (RASSF1A) and glutathione S-transferase P1 (GSTP1).

RASSF1A

Hypermethylation of *RASSF1A* is a common phenomenon in breast, kidney, liver, lung and prostate cancer. *RASSF1A* hypermethylation has been observed in 60–70% of prostate tumors, demonstrating a clear association with aggressive prostate tumors. No *RASSF1A* hypermethylation has been detected in normal prostate tissue [63,64]. These findings suggest that RASSF1A hypermethylation may distinguish between the more aggressive and indolent tumors (TABLE 2). Further studies are needed to assess its diagnostic value.

GSTP1

The most thoroughly described epigenetic alteration in prostate cancer is the hypermethylation of the *GSTP1* promoter. *GSTP1* belongs to the cellular protection system against toxic effects and as such, is involved in the detoxification of many xenobiotics. *GSTP1* hypermethylation has been reported in approximately 6% of proliferative inflammatory atrophy (PIA) lesions and in 70% of PIN lesions [65]. It has been shown that some PIA lesions merge directly with PIN and early carcinoma lesions, although additional studies are necessary to confirm these findings. Hypermethylation of *GSTP1* has been detected in more than 90% of prostate tumors, whereas no hypermethylation has been observed in BPH and normal prostate tissues [66]. In another study, hypermethylation of the *GSTP1* gene has been detected in 50% of ejaculates from prostate cancer patients but not in men with BPH. Due to the fact that ejaculates are not always easily obtained from prostate cancer patients, hypermethylation of *GSTP1* was determined in urinary sediments obtained from prostate cancer patients after prostate massage. Cancer could be detected in 77% of these sediments [67]. Moreover, hypermethylation of *GSTP1* has been found in urinary sediments after prostate massage in 68% of patients with early confined disease, 78% of patients with locally advanced disease, 29% of patients with PIN and 2% of patients with BPH. These findings resulted in a specificity of 98% and a sensitivity of 73% [68]. The negative predictive value of this test was 80%, which shows that this assay bears great potential to reduce the number of unnecessary biopsies.

Recently, *GSTP1* hypermethylation has been detected in 40–50% of urinary sediments that were obtained from patients who recently underwent prostate biopsies. *GSTP1* hypermethylation was detected in urinary sediments of patients with negative biopsies (33%) and patients with atypia or high-grade PIN (67%). Since hypermethylation of *GSTP1* has a high specificity for prostate cancer, it suggests that these patients may have occult prostate cancer [69]. This indicates that the test could also be used as an indicator for a second biopsy.

Genes uniquely expressed in prostate cancer

Microarray studies have been very useful and informative in identifying genes that are consistently up- or downregulated in prostate cancer compared with benign prostate tissue [11]. These genes can provide prostate cancer-specific biomarkers and provide a greater insight into the etiology of the disease. For the molecular diagnosis of prostate cancer, genes that are highly upregulated in prostate cancer compared with low or normal expression in normal prostate tissue are of special interest. Such genes could enable the detection of one tumor cell in a huge background of normal cells and thus be applied as a diagnostic marker in prostate cancer detection.

TMPRSS2

cDNA microarray analysis in the prostate cancer cell line LNCaP has led to the discovery of the serine protease TMPRSS2, which was found to be upregulated by androgens. *In situ* hybridization studies have shown that TMPRSS2 was highly expressed in the basal cells of normal human prostate tissue and in adenocarcinoma cells. Low expression of TMPRSS2 has been found in colon, lung, kidney and pancreas. A 492-amino acid protein has been predicted for TMPRSS2. This predicted protein is a Type II integral membrane protein, most similar to the hepsin family of proteins. These proteins are important for cell growth and maintenance of cell morphology. There are speculations that TMPRSS2 could be an activator of the precursor forms of PSA and hK2 and that TMPRSS2, like other serine proteases, may play a role in prostate carcinogenesis [70]. Since TMPRSS2 has a low prostate cancer specificity, it cannot be applied in the detection of prostate cancer cells in urinary sediments.

AMACR

The gene coding for α-methylacyl-CoA racemase (AMACR) on chromosome 5p13 has been found to be consistently upregulated in prostate cancer. This enzyme plays a critical role in peroxisomal β-oxidation of branched chain fatty acid molecules obtained from dairy and beef [71]. Interestingly, the consumption of dairy and beef has been associated with an increased risk for prostate cancer [72].

In clinical prostate cancer tissue, a ninefold overexpression of AMACR mRNA has been found compared with normal prostate tissue. IHC studies and western blot analyses have confirmed an upregulation of AMACR at the protein level. Furthermore, it has been shown that 88% of prostate cancer cases and both untreated metastases and hormone-refractory prostate cancers were strongly positive for AMACR [73]. AMACR expression has not been detected in atrophic glands, basal cell hyperplasia and urothelial epithelium or metaplasia. IHC studies also showed that AMACR expression in needle biopsies had a 97% sensitivity and a 100% specificity for prostate cancer detection [74]. Combined with a staining for p63, a basal cell marker absent in prostate cancer, AMACR greatly facilitated the identification of malignant prostate cells. Its high expression and cancer cell specificity implies that AMACR may also

be a candidate for the development of molecular probes that may facilitate the identification of prostate cancer using noninvasive imaging modalities (TABLE 2) [73].

Hepsin

Using cDNA microarray analysis, it has been shown that hepsin, a Type II transmembrane serine protease, is one of the most differentially overexpressed genes in prostate cancer compared with normal prostate and BPH tissues [39,75,76]. Using quantitative real-rime PCR analysis, it has been shown that hepsin is overexpressed in 90% of prostate cancer tissues. In 59% of prostate cancers, this overexpression was more than tenfold. There has also been a significant correlation between the upregulation of hepsin and tumor grade. Further studies must determine the tissue specificity of hepsin and the diagnostic value of this serine protease as a new serum marker. Since hepsin is upregulated in advanced and more aggressive tumors, a role as a prognostic tissue marker is suggested in determining the aggressiveness of a tumor (TABLE 2) [77].

Telomerase

Telomerase, a ribonucleoprotein, is involved in the synthesis and repair of telomeres that cap and protect the ends of eukaryotic chromosomes. The human telomeres consist of tandem repeats of the TTAGGG sequence as well as several different binding proteins. During cell division, telomeres cannot be fully replicated and become shorter. Telomerase can lengthen the telomeres and thus prevents the shortening of these structures. Cell division in the absence of telomerase activity will lead to shortening of the telomeres. As a result, the lifespan of the cell becomes limited and this will lead to senescence and cell death [78].

In tumor cells, including prostate cancer cells, telomeres are significantly shorter than in normal cells. In cancer cells with short telomeres, telomerase activity is required to escape senescence and to allow immortal growth. High telomerase activity has been found in 90% of prostate cancers and was shown to be absent in normal prostate tissue. In a small study on 36 specimens, telomerase activity has been used to detect prostate cancer cells in voided urine or urethral washing after prostate massage. This test had a sensitivity of 58% and a specificity of 100% [79]. The negative predictive value of the test was 55%. Although it was a small and preliminary study, the low negative predictive value indicates that telomerase activity measured in urine samples is not very effective in reducing the number of unnecessary biopsies.

The quantification of the catalytic subunit of telomerase, hTERT, showed a median overexpression of hTERT mRNA of sixfold in prostate cancer tissues compared with normal prostate tissues. A significant relationship was found between hTERT expression and tumor stage but not with Gleason score. The quantification of hTERT using real-time PCR showed that hTERT could accurately discriminate between prostate cancer and nonmalignant prostate tissue. However, hTERT mRNA is expressed in leukocytes, which are regularly present in body fluids such as blood and urine [80]. This may cause false positivity. As such, quantitative measurement of hTERT in body fluids is not very promising as a diagnostic tool for prostate cancer.

PSMA

Prostate-specific membrane antigen (PSMA) is a transmembrane glycoprotein that is expressed on the surface of prostate epithelial cells. The expression of PSMA appears to be restricted to the prostate. It has been shown that PSMA is upregulated in prostate cancer tissue compared with benign prostate tissues. No overlap in PSMA expression has been found between BPH and prostate cancer, indicating that PSMA is a very promising diagnostic marker (TABLE 2) [81]. Recently, it has been shown that high PSMA expression in prostate cancer cases correlated with tumor grade, pathological stage, aneuploidy and biochemical recurrence. Furthermore, increased PSMA mRNA expression in primary prostate cancers and metastasis correlated with PSMA protein overexpression [82]. Its clinical utility as a diagnostic or prognostic marker for prostate cancer has been hindered by the lack of a sensitive immunoassay for this protein. However, a combination of ProteinChip® (Ciphergen Biosystems) arrays and SELDI-TOF MS has led to the introduction of a protein biochip immunoassay for the quantification of serum PSMA. It was shown that the average serum PSMA levels for prostate cancer patients were significantly higher compared with those of men with BPH and healthy controls [83]. These findings implicate a role for serum PSMA to distinguish men with BPH from prostate cancer patients. However, further studies are needed to assess its diagnostic value.

RT-PCR studies have shown that PSMA in combination with its splice variant PSMA´ could be used as a prognostic marker for prostate cancer. In the normal prostate, PSMA´ expression is higher than PSMA expression. In prostate cancer tissues, the PSMA expression is more dominant. Therefore, the ratio of PSMA to PSMA´ is highly indicative of disease progression. Designing a quantitative PCR analysis that discriminates between the two PSMA forms could yield another application for PSMA in diagnosis and prognosis of prostate cancer (TABLE 2) [81].

δ-catenin

δ-catenin (p120/CAS), an adhesive junction-associated protein, has been shown to be highly discriminative between BPH and prostate cancer. *In situ* hybridization studies showed the highest expression of δ-catenin transcripts in adenocarcinoma of the prostate and low to no expression in BPH tissue. The average overexpression of δ-catenin in prostate cancer compared with BPH is 15.7-fold. Neither quantitative PCR nor *in situ* hybridization analysis could find a correlation between δ-catenin expression and Gleason score [81]. Further studies are needed to assess the tissue specificity and diagnostic value of δ-catenin. However, it is clear that it has limitations when used as a prognostic marker for prostate cancer.

DD3^{PCA3}

DD3^{PCA3} has been identified using differential display analysis. DD3^{PCA3} was found to be highly overexpressed in prostate tumors compared with normal prostate tissue of the same patient using northern blot analysis [84]. Moreover, DD3^{PCA3} was found to be strongly overexpressed in more than 95% of primary prostate cancer specimens and prostate cancer metastases. Furthermore, expression of DD3^{PCA3} is restricted to prostatic tissue, that is, no expression has been found in other normal human tissues [80,85]. The gene encoding DD3^{PCA3} is located on chromosome 9q21.2. The DD3^{PCA3} mRNA contains a high density of stop codons. Therefore, it lacks an open reading frame, resulting in noncoding RNA. Recently, a time-resolved quantitative RT-PCR assay (using an internal standard and an external calibration curve) has been developed. The accurate quantification power of this assay showed a median 66-fold upregulation of DD3^{PCA3} in prostate cancer tissue compared with normal prostate tissue. Moreover, a median upregulation of 11-fold was found in prostate tissues containing less than 10% of prostate cancer cells. This indicated that DD3^{PCA3} was capable of detecting a small number of tumor cells in a huge background of normal cells.

This hypothesis has been tested using the quantitative RT-PCR analysis on voided urine samples. PSA mRNA expression was shown to be relatively constant in normal prostate cells and only a weak downregulation (~1.5-fold) of PSA expression has been reported in prostate cancer cells. Therefore, PSA mRNA has been used as a housekeeping gene to correct for the number of prostate cells present in urinary sediments. These urine samples were obtained following extensive prostate massage from a group of 108 men who were indicated for prostate biopsies based on a total serum PSA value of more than 3 ng/ml. This test had 67% sensitivity and 83% specificity using prostatic biopsies as a gold standard for the presence of a tumor. Furthermore, this test had a negative predictive value of 90%, which indicates that the quantitative determination of DD3^{PCA3} transcripts in urinary sediments obtained after extensive prostate massage bears great potential in the reduction of the number of invasive TRUS-guided biopsies in this population of men [85].

The tissue specificity and the high overexpression in prostate tumors indicate that DD3^{PCA3} is the most prostate cancer-specific gene described so far. Therefore, validated DD3^{PCA3} assays could become valuable in the detection of disseminated prostate cancer cells in serum or plasma [86]. Multicenter studies using the validated DD3^{PCA3} assay can provide the basis for molecular diagnostics in clinical urological practice (TABLES 1 & 2).

RT-PCR-based detection of circulating tumor cells

With the introduction of highly sensitive PCR technology, the detection of a single tumor cell in the huge background of predominantly normal cells became feasible to improve cancer diagnosis in blood samples. It is assumed that transcripts of epithelial cells do not normally occur in the blood circulation. Therefore, the detection of these transcripts in the serum or plasma indicates the presence of disseminated prostate cancer cells. In the last 12 years, many reports have been written on the RT-PCR-based detection of disseminated prostate cancer cells using PSA mRNA as the target. However, remarkable differences were observed in the sensitivity of the RT-PCR-based assays since these assays were qualitative, not standardized and difficult to reproduce in various laboratories [87]. To enhance the sensitivity of these assays, researchers used nested PCR. This led to the amplification of illegitimate transcripts [88]. These are transcripts that have been produced and secreted in small amounts by any normal cell in the body, such as normal blood or epithelial cells. As a result, PSA mRNA transcripts were found in the serum of women and healthy controls [89]. As such, these RT-PCR-based methods were of limited value. New sensitive, quantitative and more reproducible assays using exogenous internal standards for the detection of PSA and hK2 mRNA transcripts overcame this problem [90]. However, another problem arose when using organ-specific and not cancer-specific transcripts, such as PSA or hK2 mRNA. PSA mRNA transcripts were detected in the serum or plasma of men with and without prostate cancer after prostate biopsies. This led to a false-positive indication for the presence of a disseminated cancer cell [91,92]. However, the identification of highly overexpressed prostate cancer-specific genes combined with the validated quantitative RT-PCR assays could become valuable in the detection of disseminated cancer cells in serum or plasma.

Conclusions

Prostate cancer is a very sneaky disease since it develops within and even outside the prostate before an initial diagnosis is made. Since there are no adequate therapeutic options for advanced prostate cancer, it is imperative that it is detected at an early stage when it is potentially curable. The screening tests for PSA show high sensitivity for prostate cancer detection. However, in the diagnostic gray zone, the specificity of PSA is only 20%, resulting in a negative biopsy rate of 70–80%. It has become clear that new markers are urgently needed to improve the specificity of PSA in the diagnostic gray zone.

Innovations in the field of molecular technology are rapidly growing. Recent technologies such as microarrays and proteomics have already provided new markers. In this review, several markers have been discussed that might have clinical utility in the early diagnosis of prostate cancer. The number of serum markers is rapidly growing due to the extensive human kallikrein family. By themselves they will not be able to improve the specificity for the early detection of prostate cancer. However, combined as a panel of markers, the specificity can be remarkably improved. Since prostate cancer is a heterogeneous disease, it becomes clear that a defined set of markers will become important in early prostate cancer diagnosis. Novel tests based on GSTP1 hypermethylation and the DD3^{PCA3} gene, which is highly overexpressed in prostate cancer, enabled the noninvasive detection of prostate cancer in body fluids such as urine or ejaculates. It becomes clear that through evaluation and clinical testing of the markers described herein, a greater insight into their true diagnostic potential emerges.

Expert opinion

The application of new technologies has shown that a large number of genes are upregulated in prostate cancer. For non-invasive screening tests, only those genes that are over-expressed in more than 95% of prostate cancer tissues compared with normal prostate or BPH will be important. Moreover, the upregulation of these genes in cancer should be more than tenfold in prostate cancer compared with normal prostate to enable the detection of a single prostate cancer cell in a huge background of normal cells in body fluids. The close collaboration and communication between clinicians and researchers is essential in clinical testing of these markers to assess their true diagnostic potential and to evaluate the impact of these tests on the reduction of unnecessary biopsies and disease mortality.

Five-year view

Despite the success of free PSA in cancer detection, several limitations remain. In the coming years, novel assays will be developed for the measurement of the distinct forms of serum free PSA, particularly proPSA. Only evaluation of these parameters in clinical trials will demonstrate their applicability in prostate cancer detection and lead to a reduction in the number of unnecessary biopsies.

The combination of hK2 with several forms of PSA offers promising approaches for the discrimination of prostate cancer patients from men with BPH, and may be used in the prediction of organ-confined disease. Clinical studies using large patient groups will demonstrate the applicability of hK11 in the reduction of the number of unnecessary biopsies. The combination of %fPSA values with hK11/tPSA ratios appears especially promising. The application of artificial neural networks and various logistic regression models may aid in the improvement of the positive predictive value of these serum markers. Large multicenter studies will show the real diagnostic application of these kallikreins and may lead to promising new serum-based tests. Another challenge of the coming years

is to unravel the biological and physiological functions of hK4, hK11, KLK14 and KLK15.

In the next few years, the number of potential biomarkers will grow substantially, as will our understanding of the etiology of prostate cancer. For diagnostic purposes, it is very important that the potential biomarkers are tested in terms of tissue specificity and discrimination potential between prostate cancer, normal prostate and BPH. Until now, only telomerase, GSTP1 and DD3^{PCA3} have been studied for their potential to reduce the number of biopsies. Ironically, TRUS-guided prostate biopsies are used as the gold standard in these studies. Therefore, many of the patients who are currently regarded as being negative for prostate cancer may become cancer patients in the near future. Consequently, follow-up data of these clinical studies will become very important to achieve improved outcomes and management of prostate cancer.

To date, the most prostate cancer-specific gene is *DD3^{PCA3}*. Its potential to reduce the number of biopsies in men with PSA values between 3 and 10 ng/ml is currently being validated in a large multicenter trial. A clinically applicable assay that will be reproducible within and between laboratories, which can be used as a reflex test following total PSA testing, is also under development. This urinary DD3^{PCA3} test will soon become available to urologists.

Innovations in new technologies will enable the development of more sensitive and accurate marker-based tests. Proteomic analysis and TMA will become increasingly important in discovering a pattern of proteins or genes which can discriminate between prostate cancer patients and men with BPH. These analyses may also indicate men at risk for progression of disease. Validated quantitative RT-PCR assays that are based on the detection of truly prostate cancer-specific genes may become important for the identification of patients with malignant disease.

Noninvasive marker-based assays used as screening tests in the early detection of prostate cancer are rapidly becoming reality.

Key issues

- Early detection of prostate cancer when it is still organ confined can increase the cure rate and prevent mortality from this disease.

- Currently, the low specificity of prostate-specific antigen (PSA) leads to 70–80% of unnecessary biopsies in the diagnostic PSA gray zone of 3–10 ng/ml.

- Recent developments in the field of molecular techniques have provided new tools that have led to the discovery of many new promising biomarkers for prostate cancer. These biomarkers may be instrumental in the development of new screening tests that have a high specificity in the diagnostic gray zone and as such are able to reduce the number of unnecessary biopsies.

- For diagnostic purposes, it is important that the potential biomarkers are tested in terms of tissue specificity and discrimination potential between prostate cancer, normal prostate and benign prostatic hyperplasia.

- The results of multiple marker-based assays may enhance the specificity for cancer detection in the diagnostic gray zone and may discriminate between more aggressive and indolent tumors.

References

Papers of special note have been highlighted as:
• of interest
•• of considerable interest

1   Jensen OM, Esteve J, Moller H *et al.*
     Cancer in the European Community and
     its member states. *Eur. J. Cancer* 26,
     1167–1256 (1990).

2   Jemal A, Tiwari RC, Murray T *et al.*
     Cancer statistics, 2004. *CA Cancer J. Clin.*
     54, 8–29 (2004).

3   Schroder FH, van der Maas P,
     Beemsterboer P *et al.* Evaluation of the
     digital rectal examination as a screening test
     for prostate cancer. Rotterdam section of
     the European randomized study of
     screening for prostate cancer. *J. Natl Cancer
     Inst.* 90, 1817–1823 (1998).

4   Schroder FH. Diagnosis, characterization
     and potential clinical relevance of prostate
     cancer detected at low PSA ranges.
     Eur. Urol. 39(Suppl. 4), 49–53 (2001).

5   Catalona WJ, Richie JP, Ahmann FR *et al.*
     Comparison of digital rectal examination
     and serum prostate-specific antigen in the
     early detection of prostate cancer: results of
     a multicenter clinical trial of 6630 men.
     J. Urol. 151, 1283–1290 (1994).

6   Djavan B, Zlotta A, Remzi M *et al.*
     Optimal predictors of prostate cancer on
     repeat prostate biopsy: a prospective study
     of 1051 men. *J. Urol.* 163, 1144–1148
     (2000).

7   Partin AW, Oesterling JE. The clinical
     usefulness of prostate-specific antigen:
     update 1994. *J. Urol.* 152, 1358–1368
     (1994).

8   Etzioni R, Urban N, Ramsey S *et al.*
     The case for early detection. *Nature Rev.
     Cancer* 3, 243–252 (2003).
••   **Provides a rationale and plan for early
     detection research.**

9   Montironi R, Mazzucchelli R, Scarpelli M.
     Molecular techniques and prostate cancer
     diagnostic. *Eur. Urol.* 44, 390–400
     (2003).
•   **Recently developed molecular techniques
     are described and illustrated. Focus is
     placed on the applicability of these
     techniques in the diagnosis of
     prostate cancer.**

10   Petricoin EF, Liotta LA. Proteomic analysis
     at the bedside: early detection of cancer.
     Trends Biotechnol. 20, S30–S34 (2002).

11   Kumar-Sinha C, Rhodes DR, Yu J *et al.*
     Prostate cancer biomarkers: a current
     perspective. *Expert Rev. Mol. Diagn.* 3,
     459–470 (2003).
••   **Recently discovered genes are described.**

12   Lichtenstein P, Holm NV, Verkasalo PK
     et al. Environmental and heritable factors
     in the causation of cancer-analyses of
     cohorts of twins from Sweden, Denmark
     and Finland. *N. Engl. J. Med.* 343, 78–85
     (2000).

13   Monroe KR, Yu MC, Kolonel LN *et al.*
     Evidence of an X-linked or recessive genetic
     component to prostate cancer risk. *Nature
     Med.* 1, 827–829 (1995).

14   Simard J, Dumont M, Labuda D *et al.*
     Prostate cancer susceptibility genes: lessons
     learned and challenges posed. *Endocr.
     Relat. Cancer* 10, 225–259 (2003).

15   Nam RK, Zhang WW, Trachtenberg J *et al.*
     Comprehensive assessment of candidate
     genes and serological markers for the
     detection of prostate cancer. *Cancer
     Epidemiol. Biomarkers Prev.* 12,
     1429–1437 (2003).

16   Bookstein R, MacGrogan D,
     Hilsenbeck SG *et al.* p53 is mutated in a
     subset of advanced-stage prostate cancers.
     Cancer Res. 53, 3369–3373 (1993).

17   Navone NM, Troncoso P, Pisters LL *et al.*
     p53 protein accumulation and gene
     mutation in the progression of human
     prostate carcinoma. *J. Natl Cancer Inst.*
     20(85), 1657–1669 (1993).

18   Fliss MS, Usadel H, Caballero OL *et al.*
     Facile detection of mitochondrial DNA
     mutations in tumors and bodily fluids.
     Science 287, 2017–2019 (2000).

19   Jeronimo C, Nomoto S, Caballero OL *et al.*
     Mitochondrial mutations in early stage
     prostate cancer and bodily fluids. *Oncogene*
     20, 5195–5198 (2001).

20   Goessl C, Muller M, Straub B *et al.*
     DNA alterations in body fluids as
     molecular tumor markers for urological
     malignancies. *Eur. Urol.* 41, 668–676
     (2002).

21   Yousef GM, Diamandis EP. Human tissue
     kallikreins: a new enzymatic cascade
     pathway? *Biol. Chem.* 383, 1045–1057
     (2002).

22   Diamandis EP, Yousef GM. Human tissue
     kallikrein gene family: a rich source of novel
     disease biomarkers. *Expert Rev. Mol. Diagn.*
     1, 182–190 (2001).

23   Meng FJ, Shan A, Jin L *et al.*
     The expression of a variant prostate-specific
     antigen in human prostate. *Cancer
     Epidemiol. Biomarkers Prev.* 11, 305–309
     (2002).

24   Stamey TA, Yang N, Hay AR *et al.*
     Prostate-specific antigen as a serum marker
     for adenocarcinoma of the prostate.
     N. Engl. J. Med. 317, 909–916
     (1987).

25   Nixon RG, Brawer MK. Enhancing the
     specificity of prostate-specific antigen
     (PSA): an overview of PSA density, velocity
     and age-specific reference ranges.
     Br. J. Urol. 79(Suppl. 1), 61–67
     (1997).

26   Ukimura O, Durrani O, Babaian RJ.
     Role of PSA and its indices in determining
     the need for repeat prostate biopsies.
     Urology 50, 66–72 (1997).

27   Catalona WJ, Smith DS, Wolfert RL *et al.*
     Evaluation of percentage of free serum
     prostate-specific antigen to improve
     specificity of prostate cancer screening.
     JAMA 274, 1214–1220 (1995).

28   Catalona WJ, Partin AW, Slawin KM *et al.*
     Use of the percentage of free prostate-
     specific antigen to enhance differentiation
     of prostate cancer from benign prostatic
     disease: a prospective multicenter clinical
     trial. *JAMA* 279(19), 1542–1547
     (1998).

29   Jung K, Meyer A, Lein M *et al.* Ratio of
     free-to-total prostate-specific antigen in
     serum cannot distinguish patients with
     prostate cancer from those with chronic
     inflammation of the prostate. *J. Urol.* 159,
     1595–1598 (1998).

30   Murphy GP, Barren RJ, Erickson SJ *et al.*
     Evaluation and comparison of two new
     prostate carcinoma markers. Free-prostate-
     specific antigen and prostate specific
     membrane antigen. *Cancer* 78, 809–818
     (1996).

31   Bangma CH, Rietbergen JB, Kranse R *et al.*
     The free-to-total prostate-specific antigen
     ratio improves the specificity of prostate-
     specific antigen in screening for prostate
     cancer in the general population. *J. Urol.*
     157, 2191–2196 (1997).

32   Wald NJ, Watt HC, George L *et al.*
     Adding free to total prostate-specific
     antigen levels in trials of prostate cancer
     screening. *Br. J. Cancer* 82, 731–736
     (2000).

33   Mikolajczyk SD, Marks LS, Partin AW
     et al. Free prostate-specific antigen in serum
     is becoming more complex. *Urology* 59,
     797–802 (2002).

34   Mikolajczyk SD, Rittenhouse HG.
     ProPSA: a more cancer specific form of
     prostate-specific antigen for the early
     detection of prostate cancer. *Keio J. Med.*
     52, 86–91 (2003).

35   Khan MA, Partin AW, Rittenhouse HG
     et al. Evaluation of proprostate-specific
     antigen for early detection of prostate
     cancer in men with a total prostate-specific
     antigen range of 4.0 to 10.0 ng/ml. *J. Urol.*
     170, 723–726 (2003).

36   Mikolajczyk SD, Catalona WJ, Evans CL *et al.* Proenzyme forms of prostate-specific antigen in serum improve the detection of prostate cancer. *Clin. Chem.* 50(6), 1017–1025 (2004).

37   Stephan C, Jung K, Lein M *et al.* Molecular forms of prostate-specific antigen and human kallikrein 2 as promising tools for early diagnosis of prostate cancer. *Cancer Epidemiol. Biomarkers Prev.* 9, 1133–1147 (2000).

•   Extensive overview on the molecular forms of prostate-specific antigen (PSA) and hK2 and their applicability in the early diagnosis of prostate cancer.

38   Magklara A, Scorilas A, Stephan C *et al.* Decreased concentrations of prostate-specific antigen and human glandular kallikrein 2 in malignant versus nonmalignant prostatic tissue. *Urology* 56, 527–532 (2000).

39   Stamey TA, Warrington JA, Caldwell MC *et al.* Molecular genetic profiling of Gleason grade 4/5 prostate cancers compared to benign prostatic hyperplasia. *J. Urol.* 166, 2171–2177 (2001).

40   Becker C, Piironen T, Pettersson K *et al.* Discrimination of men with prostate cancer from those with benign disease by measurements of human glandular kallikrein 2 (HK2) in serum. *J. Urol.* 163, 311–316 (2000).

41   Kwiatkowski MK, Recker F, Piironen T *et al.* In prostatism patients the ratio of human glandular kallikrein to free PSA improves the discrimination between prostate cancer and benign hyperplasia within the diagnostic 'gray zone' of total PSA 4 to 10 ng/ml. *Urology* 52, 360–365 (1998).

42   Recker F, Kwiatkowski MK, Piironen T *et al.* Human glandular kallikrein as a tool to improve discrimination of poorly differentiated and non-organ-confined prostate cancer compared with prostate-specific antigen. *Urology* 55, 481–485 (2000).

43   Klee GG, Goodmanson MK, Jacobsen SJ *et al.* Highly sensitive automated chemiluminometric assay for measuring free human glandular kallikrein-2. *Clin. Chem.* 45, 800–806 (1999).

44   Haese A, Graefen M, Steuber T *et al.* Human glandular kallikrein 2 levels in serum for discrimination of pathologically organ-confined from locally-advanced prostate cancer in total PSA-levels below 10 ng/ml. *Prostate* 49, 101–109 (2001).

45   Nelson PS, Gan L, Ferguson C *et al.* Molecular cloning and characterization of prostase, an androgen-regulated serine protease with prostate-restricted expression. *Proc. Natl Acad. Sci. USA* 96, 3114–3119 (1999).

46   Yousef GM, Obiezu CV, Luo LY *et al.* Prostase/KLK-L1 is a new member of the human kallikrein gene family, is expressed in prostate and breast tissues and is hormonally regulated. *Cancer Res.* 59, 4252–4256 (1999).

47   Day CH, Fanger GR, Retter MW *et al.* Characterization of KLK4 expression and detection of KLK4-specific antibody in prostate cancer patient sera. *Oncogene* 21, 7114–7120 (2002).

48   Obiezu CV, Soosaipillai A, Jung K *et al.* Detection of human kallikrein 4 in healthy and cancerous prostatic tissues by immunofluorometry and immunohistochemistry. *Clin. Chem.* 48, 1232–1240 (2002).

49   Xi Z, Klokk TI, Korkmaz K *et al.* Kallikrein 4 is a predominantly nuclear protein and is overexpressed in prostate cancer. *Cancer Res.* 64, 2365–2370 (2004).

50   Diamandis EP, Okui A, Mitsui S *et al.* Human kallikrein 11: a new biomarker of prostate and ovarian carcinoma. *Cancer Res.* 62, 295–300 (2002).

51   Nakamura T, Scorilas A, Stephan C *et al.* The usefulness of serum human kallikrein 11 for discriminating between prostate cancer and benign prostatic hyperplasia. *Cancer Res.* 63, 6543–6546 (2003).

•   Demonstrates the diagnostic potential of the combination of hK11, percent free PSA and total PSA in the reduction of prostate biopsies.

52   Yousef GM, Magklara A, Chang A *et al.* Cloning of a new member of the human kallikrein gene family, KLK14, which is downregulated in different malignancies. *Cancer Res.* 61, 3425–3431 (2001).

53   Hooper JD, Bui LT, Rae FK *et al.* Identification and characterization of KLK14, a novel kallikrein serine protease gene located on human chromosome 19q13.4 and expressed in prostate and skeletal muscle. *Genomics* 73, 117–122 (2001).

54   Yousef GM, Stephan C, Scorilas A *et al.* Differential expression of the human kallikrein gene 14 (KLK14) in normal and cancerous prostatic tissues. *Prostate* 56, 287–292 (2003).

55   Yousef GM, Scorilas A, Jung K *et al.* Molecular cloning of the human kallikrein 15 gene (KLK15). Upregulation in prostate cancer. *J. Biol. Chem.* 276, 53–61 (2001).

56   Stephan C, Yousef GM, Scorilas A *et al.* Quantitative analysis of kallikrein 15 gene expression in prostate tissue. *J. Urol.* 169, 361–364 (2003).

57   Hlavaty JJ, Partin AW, Shue MJ *et al.* Identification and preliminary clinical evaluation of a 50.8-kDa serum marker for prostate cancer. *Urology* 61, 1261–1265 (2003).

58   Banez LL, Prasanna P, Sun L *et al.* Diagnostic potential of serum proteomic patterns in prostate cancer. *J. Urol.* 170, 442–446 (2003).

•   Demonstrates that complex serum protein profiles have the potential to identify prostate cancer and could be applied as a new tool for prostate cancer diagnosis.

59   Petricoin EF, Ardekani AM, Hitt BA *et al.* Use of proteomic patterns in serum to identify ovarian cancer. *Lancet* 359, 572–577 (2002).

60   Li S, Hursting SD, Davis BJ *et al.* Environmental exposure, DNA methylation and gene regulation: lessons from diethylstilbesterol-induced cancers. *Ann. NY Acad. Sci.* 983, 161–169 (2003).

61   Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. *Nature Rev. Genet.* 3, 415–428 (2002).

62   Goessl C, Krause H, Muller M *et al.* Fluorescent methylation-specific polymerase chain reaction for DNA-based detection of prostate cancer in bodily fluids. *Cancer Res.* 60, 5941–5945 (2000).

63   Liu L, Yoon JH, Dammann R *et al.* Frequent hypermethylation of the RASSF1A gene in prostate cancer. *Oncogene* 21, 6835–6840 (2002).

64   Kuzmin I, Gillespie JW, Protopopov A *et al.* The RASSF1A tumor suppressor gene is inactivated in prostate tumors and suppresses growth of prostate carcinoma cells. *Cancer Res.* 62, 3498–3502 (2002).

65   Nakayama M, Bennett CJ, Hicks JL *et al.* Hypermethylation of the human glutathione S-transferase-P1 gene (GSTP1) CpG island is present in a subset of proliferative inflammatory atrophy lesions but not in normal or hyperplastic epithelium of the prostate: a detailed study using laser-capture microdissection. *Am. J. Pathol.* 163, 923–933 (2003).

66   Lee WH, Morton RA, Epstein JI *et al.* Cytidine methylation of regulatory sequences near the P1-class glutathione S-transferase gene accompanies human prostatic carcinogenesis. *Proc. Natl Acad. Sci. USA* 91, 11733–11737 (1994).

67 Goessl C, Muller M, Heicappell R *et al.*
    DNA-based detection of prostate cancer in
    blood, urine and ejaculates. *Ann. NY Acad.
    Sci.* 945, 51–58 (2001).

68 Goessl C, Muller M, Heicappell R *et al.*
    DNA-based detection of prostate cancer in
    urine after prostatic massage. *Urology* 58,
    335–338 (2001).

•   **Demonstrates that the detection of
    glutathione S-transferase P1 methylation
    can be used as a urine-based diagnostic
    tool for the detection of prostate cancer.**

69 Gonzalgo ML, Pavlovich CP, Lee SM *et al.*
    Prostate cancer detection by GSTP1
    methylation analysis of postbiopsy urine
    specimens. *Clin. Cancer Res.* 9, 2673–2677
    (2003).

70 Lin B, Ferguson C, White JT *et al.*
    Prostate-localized and androgen-regulated
    expression of the membrane-bound serine
    protease TMPRSS2. *Cancer Res.* 59,
    4180–4184 (1999).

71 Schmitz W, Albers C, Fingerhut R *et al.*
    Purification and characterization of an
    α-methylacyl-CoA racemase from human
    liver. *Eur. J. Biochem.* 231, 815–822
    (1995).

72 Michaud DS, Augustsson K, Rimm EB
    et al. A prospective study on intake of
    animal products and risk of prostate cancer.
    Cancer Causes Control 12, 557–567 (2001).

73 Luo J, Zha S, Gage WR *et al.*
    α-methylacyl-CoA racemase: a new
    molecular marker for prostate cancer.
    Cancer Res. 62, 2220–2226 (2002).

74 Rubin MA, Zhou M, Dhanasekaran SM
    et al. α-methylacyl coenzyme A racemase as
    a tissue biomarker for prostate cancer.
    JAMA 287, 1662–1670 (2002).

75 Luo J, Duggan DJ, Chen Y *et al.*
    Human prostate cancer and benign
    prostatic hyperplasia: molecular dissection
    by gene expression profiling. *Cancer Res.*
    61, 4683–4688 (2001).

76 Magee JA, Araki T, Patil S *et al.*
    Expression profiling reveals hepsin
    overexpression in prostate cancer. *Cancer
    Res.* 61, 5692–5696 (2001).

77 Stephan C, Yousef GM, Scorilas A *et al.*
    Hepsin is highly over expressed in and a
    new candidate for a prognostic indicator in
    prostate cancer. *J. Urol.* 171, 187–191
    (2004).

78 Blasco MA. Telomerase beyond telomeres.
    Nature Rev. Cancer 2, 627–633 (2002).

79 Meid FH, Gygi CM, Leisinger HJ *et al.*
    The use of telomerase activity for the
    detection of prostatic cancer cells after
    prostatic massage. *J. Urol.* 165, 1802–1805
    (2001).

80 de Kok JB, Verhaegh GW, Roelofs RW
    et al. DD3(PCA3), a very sensitive and
    specific marker to detect prostate tumors.
    Cancer Res. 62, 2695–2698 (2002).

81 Burger MJ, Tebay MA, Keith PA *et al.*
    Expression analysis of δ-catenin and
    prostate-specific membrane antigen: their
    potential as diagnostic markers for prostate
    cancer. *Int. J. Cancer* 100, 228–237 (2002).

82 Ross JS, Sheehan CE, Fisher HA *et al.*
    Correlation of primary tumor prostate-
    specific membrane antigen expression with
    disease recurrence in prostate cancer.
    Clin. Cancer Res. 9, 6357–6362 (2003).

83 Xiao Z, Adam BL, Cazares LH *et al.*
    Quantitation of serum prostate-specific
    membrane antigen by a novel protein
    biochip immunoassay discriminates benign
    from malignant prostate disease. *Cancer
    Res.* 61, 6029–6033 (2001).

84 Bussemakers MJ, van Bokhoven A,
    Verhaegh GW *et al.* DD3: a new prostate-
    specific gene, highly overexpressed in
    prostate cancer. *Cancer Res.* 59, 5975–5979
    (1999).

85 Hessels D, Klein Gunnewiek JM,
    van Oort I *et al.* DD3(PCA3)-based
    molecular urine analysis for the diagnosis of
    prostate cancer. *Eur. Urol.* 44, 8–15 (2003).

•• **Introduces the DD3[PCA3]-based assay for
    the detection of prostate cancer cells in
    urine sediments.**

86 Oliveira JDD, Goulart LR, Waldesse PO
    et al. DD3 gene expression in peripheral
    blood of patients with prostate cancer and
    with benign hyperplasia as a molecular
    diagnostic. *5th World Congress Urol. Res.*
    London, UK (2003).

87 de la Taille A, Olsson CA, Katz AE.
    Molecular staging of prostate cancer: dream
    or reality? *Oncology (Huntingt.)* 13,
    187–194 (1999).

88 Smith MR, Biggar S, Hussain M.
    Prostate-specific antigen messenger RNA is
    expressed in non-prostate cells: implications
    for detection of micrometastases. *Cancer
    Res.* 55, 2640–2644 (1995).

89 Henke W, Jung M, Jung K *et al.* Increased
    analytical sensitivity of RT-PCR of PSA
    mRNA decreases diagnostic specificity of
    detection of prostatic cells in blood.
    Int. J. Cancer 70, 52–56 (1997).

90 Ylikoski A, Pettersson K, Nurmi J *et al.*
    Simultaneous quantification of prostate-
    specific antigen and human glandular
    kallikrein 2 mRNA in blood samples from
    patients with prostate cancer and benign
    disease. *Clin. Chem.* 48, 1265–1271
    (2002).

91 Moreno JG, O'Hara SM, Long JP *et al.*
    Transrectal ultrasound-guided biopsy causes
    hematogenous dissemination of prostate
    cells as determined by RT-PCR. *Urology* 49,
    515–520 (1997).

92 Polascik TJ, Wang ZP, Shue M *et al.*
    Influence of sextant prostate needle biopsy
    or surgery on the detection and harvest of
    intact circulating prostate cancer cells.
    J. Urol. 162, 749–752 (1999).

Affiliations

•   *Daphne Hessels
    Senior Research Technician, Experimental
    Urology, Nijmegen Center for Molecular Life
    Sciences, 6500 HB Nijmegen, The Netherlands
    Tel.: +31 24 361 0511
    Fax: +31 24 354 1222
    D.Hessels@uro.umcn.nl*

•   *Gerald W Verhaegh, PhD
    Research Scientist, Experimental Urology,
    Nijmegen Center for Molecular Life Sciences,
    6500 HB Nijmegen, The Netherlands
    Tel.: +31 243 610 510
    Fax: +31 243 541 222
    G.Verhaegh@uro.umcn.nl*

•   *Jack A Schalken, PhD
    Professor, Experimental Urology, Nijmegen
    Center for Molecular Life Sciences, 6500 HB
    Nijmegen, The Netherlands
    Tel.: +31 24 361 4146
    Fax: +31 24 354 1222
    J.Schalken@uro.umcn.nl*

•   *J Alfred Witjes, MD, PhD
    Professor, Department of Urology, University
    Medical Center Nijmegen,
    6525 GA Nijmegen, The Netherlands
    Tel.: +31 24 361 6712
    Fax: +31 24 354 1031
    F.Witjes@uro.umcn.n*