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Molecular Diagnostics in Prostate Cancer

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Abstract

Since there are no effective therapeutic options for advanced prostate cancer, early detection of this tumour is pivotal and can increase the curative success rate. Although the routine use of serum PSA testing has undoubtedly increased prostate cancer detection, one of its main drawbacks has been the lack of specificity that results in a high negative biopsy rate. Since prostate cancer is a heterogeneous disease, it has become clear that a defined set of markers will provide significantly more diagnostic information than any one biomarker. The list of potential prostate cancer biomarkers will continue to grow. Only when research groups use the proposed guidelines for biomarker development, then systematic evaluation and clinical investigation of these biomarkers will gain more insight into their true diagnostic potential.

Keywords: Prostate cancer; Diagnosis; Prognosis; Molecular techniques; Molecular tumor markers; Gene expression; Body fluids; Diagnostic tests

1. Introduction

In many developed countries, prostate cancer is the second leading cause of cancer-related deaths among men. The incidence of prostate cancer increases more with age than any other type of cancer [1]. Each year 232,090 men in the USA and 237,800 men in Europe are newly diagnosed with prostate cancer. Annually, around 30,350 US and 85,200 European men die from this disease [2,3]. Radical surgery 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 still confined to the prostate, is therefore pivotal. Since its first clinical application, prostate-specific antigen (PSA) has shown to be the most valuable tool in the detection, staging and monitoring of prostate cancer. Since 1997, the European Randomised Study of Screening for Prostate Cancer (ERSPC), Rotterdam section, accepted serum PSA values ≥ 3 ng/ml as the standard biopsy indication, irrespective of DRE or TRUS findings [4,5]. A large multi-centre prostate cancer screening trial showed that men with serum PSA values between 3 and 10 ng/ml most likely have clinically localized disease and would benefit from curative treatment [6]. However, only one in four men with serum PSA values between 3 and 10 ng/ml has prostate cancer upon biopsy resulting in a negative biopsy rate of 70–80%. The low specificity of the serum PSA test is a consequence of the fact that increased PSA levels have been reported in men with benign prostatic hyperplasia (BPH) and prostatitis and is not a prostate cancer-specific event. After the introduction of the PSA test prostate cancer is detected in younger men with no signs or symptoms of the disease. Concerns have arisen regarding the clinically insignificant prostate cancers that do not pose a serious life threat and, as a result, do not require therapy. Epidemiology studies indicate that prostate cancer is an indolent disease and it has been calculated that one in eight screen-detected prostate cancers will lead to the death of a patient when left untreated [7]. Over-diagnosis of clinically insignificant prostate cancers will cause over-treatment, including incontinence and impotence that are side-effects of radical surgery and radiotherapy, and will negatively

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affect the patients' quality of life. Furthermore, PSA screening fails to detect a small proportion of highly aggressive prostate cancers, that are likely to be life threatening. Therefore, 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.

Part I: General guidelines

2. Biomarker development

A new prostate cancer screening test has to be accurate with high sensitivity and specificity for prostate cancer detection, fast, inexpensive, non-invasive and be well accepted by the population targeted for screening. Furthermore, the test should detect prostate cancer when it is still confined to the prostate and distinguish the indolent from the aggressive tumors to avoid the problem of over-diagnosis [8]. The diagnostic accuracy of tests for prostate cancer can be improved through the identification of prostate cancer-specific genes. Therefore, it is necessary to study expression patterns in malignant as well as non-malignant prostate tissues. Developments in molecular techniques provided new tools that have led to the identification of hundreds of genes and proteins that are believed to be relevant for the development of prostate cancer [9]. The number of biomarker candidates is likely to increase in the near future. To provide guidance for a systematic and critical evaluation of putative biomarkers by research groups it has been proposed that biomarker development should occur in six phases [8,10]:

1. pre-clinical exploratory study
2. development of a second generation research assay
3. retrospective analysis
4. prospective analysis
5. marker commercialization
6. FDA approval

2.1. Phase one: pre-clinical exploratory study

The preclinical exploratory studies start with the identification of prostate cancer-specific genes. First generation research tests can be used for measurement of the putative biomarkers in tissue samples. One criterion is to assess how well a biomarker candidate can distinguish prostate cancer tissue from normal prostate tissue. A Receiver-operating characteristic (ROC) curve can display sensitivity and specificity of a biomarker in a quick manner. The area under an ROC curve (AUC-ROC) shows the discriminative ability of a marker. An AUC-ROC of 1.0 indicates that the biomarker has high diagnostic potential because it can excellently distinguish prostate cancer tissue from benign tissue. An AUC-ROC of 0.5 indicates that the biomarker has poor diagnostic potential.

2.2. Phase two: development of a second generation research assay

New biomarkers are revealed because of differences in expression patterns in malignant as well as non-malignant prostate tissues. However, tissue specimens cannot be used for clinical screening because they are obtained through invasive and expensive procedures. In the second phase of biomarker development a second generation research assay should be developed which is based on a specimen that does not require an invasive procedure. The ideal biomarker would be detectable in body fluids such as blood, ejaculate or urine. More than 85% of the prostate cancers occur in the peripheral zone of the prostate, and it has been shown that exfoliated cancer cells can be found in voided urine of prostate cancer patients. Especially the first portion of voided urine was found to be very useful when it was demonstrated to contain the highest concentration of prostatic and urethral secretions [11]. In case of organ confined disease, where the number of cancer cells exfoliated into the urine may be low, DRE can facilitate the detection of cancer cells in voided urine. For the patient it will be no extra burden since rectal examination is routinely performed in case prostate cancer is suspected.

To develop a research assay based on body fluid specimen two types of assays can be discriminated. These are the protein-based tests and the molecular gene-based tests. In Table 1 an overview is given on the available techniques and their applicability in the detection of proteins or nucleic acids.

2.2.1. Protein-based tests

The classical immunological protein-based tests are enzyme-linked immunosorbent assays (ELISAs) to determine proteins in the serum or urine of a patient (Table 1). These assays are often easy to establish relative to DNA or RNA-based assays. From the discovery of a secreted protein to a protein-based assay typically requires a period of 1–2 years depending on the time required to produce specific antibodies. Proteomic pattern diagnostics has been used in the past with 2-D gel electrophoresis but the use of mass spectrometry (MS) is a fairly new technique for cancer detection (Table 1). The protein pattern rather than

Table 1
Available techniques for biomarker determination in body fluids

Type of assay	Technique	Target	Disadvantage	Advantage	Specimen
Protein-based assays	ELISA	Based on a single protein		Relatively easy to establish	Body fluid
	SELDI-TOF MS [12]	Based on a pattern of proteins	Lack of standardization	Currently validated by EDRN [13,14]	
DNA-based assays [15]	Gene expression profiling	Based on expression several genes	Due to heterogeneity of prostate cancer only applicable in tissue specimens		Radical prostatectomy tissue
	Sequence analysis	Screen for cancer-specific mutations or polymorphisms	Ineffective in detection of primary prostate cancers in general male population		Tissue, body fluid
		Mitochondrial DNA alterations	Diagnostic applicability needs to be defined		Body fluid
	LOH	Microsatellite alterations	Prone to artefacts. Requires tumor to normal ratio of >0.5%		Body fluid
	MSP	Epigenetic modifications	No FDA approved technology	Sensitive method, requires tumor to normal ratio of 0.1–0.001%	Body fluid
RNA-based assays	RT-PCR	Specific mRNA	RNA degradation	FDA approved Roche Molecular systems	Tissue, Body fluid
	NASBA			FDA approved bioMérieux	
	TMA			FDA approved Gen-Probe	

individual proteins can be used to distinguish prostate cancer patients from healthy individuals. Moreover, the protein pattern can also aid to distinguish aggressive disease from latent disease. Recently, it has been shown that surface enhanced laser desorption/ionization time-of-flight (SELDI-TOF) MS combined with a pattern detecting algorithm for prostate cancer seems promising as diagnostic tool [12]. Since there is a large amount of commercial chip types available with different chemistries for ion exchange and metal affinity, each parameter should be well examined and optimized. One of the goals of the Early Detection Research Network (EDRN) of the National Cancer Institute is to coordinate research among biomarker-development and validation laboratories. Currently, the EDRN is evaluating the robustness of this technology in a validation trial [13,14].

2.2.2. *Molecular gene-based tests*

The gene-based tests can be divided into DNA-based and RNA-based tests (Table 1). The advantage of DNA is its better stability than RNA. Although there are many DNA-based techniques available, it is questionable whether they can be used as a screening tool. Currently, there are no prostate cancer susceptibility genes or loci responsible for the largest portion of prostate cancer. Therefore, screening men for the presence of prostate cancer-specific gene-mutations or polymorphisms is a time-consuming, expensive and very ineffective approach in the detection of primary prostate cancers in the general male population. Gene expression profiling is based on the identification of signatures of differentially expressed genes in prostate cancer. However, due to the multifocality and heterogeneity of prostate cancer it may be rather difficult to establish a reliable profile pattern from biopsy specimens. Tissues obtained from radical prostatectomy specimens are more elucidating. Therefore, gene expression profiling can be used for predicting outcome of disease after radical surgery rather than prostate cancer diagnosis. For the detection of microsatellite alterations at least 20% of the analyzed genomic DNA has to be obtained from tumor cells. Moreover, this technique is prone to artefacts using small amounts of DNA obtained from body fluids. Only methylation-specific PCR (MSP) for the detection of promoter hypermethylation, an epigenetic modification, can serve as a very useful technique in the detection of cancer in body fluid specimens because it requires a tumor-to normal ratio of only 0.1–0.001% (Table 1) [15].

To determine the specific expression of genes, messenger RNA (mRNA) is used as a target. The advantage of using RNA over DNA is the availability of commercially available, Food and Drug Administration (FDA) approved technologies such as Reverse Transcriptase-PCR (RT-PCR) (Roche Molecular Systems), Nucleic acid sequence-based amplification (NASBA) (bioMérieux), and Transciption-mediated amplification (TMA) (Gen-Probe), that enable the quantification of the mRNA of interest (Table 1).

In urine samples RNA is prone to degradation due to the presence of RNases and the acidic pH which leads to a decreased sensitivity of the RNA-based test. To avoid the ''garbage in, garbage out'' effect, a good clinical test relies on the robustness of the sample collection. The problem of RNA degradation by RNases can be overcome by the immediate addition of RNase inhibitors (e.g. guanidinium isothiocyanate (GITC) or chelating agents such as citrate or EDTA) and immediate cooling of the body fluid specimens upon collection. On the other hand, collection media such as PAXgene™ or PreservCyt® can be used as a fixative to preserve exfoliated cells and RNA. Thus, sampling, sample storage before transport, and transport of the samples are very important steps [16]. If these steps are not well standardized they will have a profound negative effect on the diagnostic test result.

In phase two, optimization and standardization of sample acquisition is established. Furthermore, the reproducibility of the developed research assay within and between laboratories is assessed. Like in phase one, an ROC curve is used to assess the ability of the body fluid-based biomarker assay to distinguish subjects with cancer from those without cancer in the population targeted for screening.

2.3. Phase three: retrospective analysis

In this phase of biomarker development the second generation research assay developed in phase two is used on stored body fluid specimens that were collected from a cohort that reflects the target population for screening. The biomarker levels obtained in cancer case subjects are compared with those obtained from healthy controls. The biomarkers potential in early diagnosis increases when the biomarker levels in prostate cancer patients differs significantly from those in the control group months or years before prostate cancer is diagnosed in biopsy specimens.

2.4. Phase 4: prospective analysis

In this phase of biomarker development the biomarker-based research assay is applied in the screening of men for prostate cancer with the aim of early diagnosis and treatment of the disease. In these studies the true and false positive rates of the biomarker-based research assay are calculated and the stage or characteristics of the tumors detected are described. Tumors are monitored that do occur clinically but are not detected by the research assay. False positive cases are monitored for the development of prostate cancer at a later time point. If the biomarker assay detects tumors that cannot be treated successfully, or tumors that regress spontaneously or tumors that grow very slow then men will derive little benefit from the detection by this biomarker [10]. Therefore, this phase determines whether further research using this biomarker-based test should be warranted or minimized.

2.5. Phase 5 and 6: marker commercialization and FDA approval

When the biomarker-based research assay has passed all four phases it is commercialized and used for screening in the general population. These screening studies will be used to estimate the reduction in cancer mortality afforded by the new biomarker-based screening test. The next very important step will be clinical trials that lead to FDA approval (phase 6).

In the next part of this review these proposed guidelines are used to indicate the progression of new and previously discussed biomarkers through the phases of development to a biomarker-based assay. Furthermore, this review can be considered as an update of the overview that was given 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 [15].

Part II: The current evaluation status of biomarkers

3. Phase one biomarkers

3.1. BMP-6

Bone morphogenetic proteins (BMP) are involved in new bone formation and organ development. In prostate cancer tissue the expression of both BMP-6 mRNA and protein is up-regulated compared with adjacent normal prostate tissue. BMP-6 mRNA expression was demonstrated in 95–100% of primary prostate cancers of men with prostate cancer metastases, 18–36% of men with organ-confined disease, and 85% of bone metastases [17,18]. Only 29% of skeletal metastases from other human carcinomas showed weak BMP-6 mRNA expression [18]. CpG demethylation of the BMP-6 promoter is responsible for the high expression in primary and secondary sites of advanced prostate cancers [19].

Co-expression of the proteins BMP-6, bone sialoprotein (another bone-related protein), and thymidine phosphorylase (an angiogenic factor) was observed in 90% of the radical prostatectomy specimens of men with bone metastases and only in 29% of men with curative treatment [20]. Therefore, co-expression of these proteins could identify patients who are at risk for disease progression after treatment (Table 2). Although there is a clear correlation between over-expression of

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

Goal	Biomarker	Application	Method	Reference
Discrimination of organ-confined disease from locally-advanced disease	hK2, fPSA/(tPSAxhK2)	Serum	Immunofluorometric assays	[93–95]
	PSCA	Tissue	IHC	[23]
Prostate cancer detection	AMACR	Biopsy	IHC, enzymatic activity assay	[27,28,30]
	EPCA			[42]
Prediction of G3 tumors.	hK2/fPSA, fPSA/(tPSAxhK2)	Serum	Immunofluorometric Assays	[95]
Distinguish the more aggressive tumors from the indolent ones.	Hepsin	Serum	Immunofluorometric assays	[96]
	hK2/PSA	Urine	Quantitative RT-PCR	[59]
	RASSF1A	Urine	MSP analysis	[15]
Reduction of the number of unnecessary biopsies.	50-kDa protein	Serum	Immunofluorometric	[15]
	AMACR immunereactivity		Assays	[31]
	CRISP-3			[35,38]
	hK11/tPSA			[15]
	hK2/fPSA			
	combination of tPSA,%fPSA and %sum-pro-PSA			
	PSMA		Biochip immunoassay	[15]
	EPCA	Plasma	Immunofluorometric Assay	[43]
	Telomerase activity	Urine/prostate fluid	TRAP	[71,72]
	GSTP1	Urine	MSP analysis	[15]
	$PCA3^{DD3}$	Urine	Quantitative RT-PCR/$uPM3^{TM}$ test	[74–76]
	AMACR protein	Urine		[97]
Prediction of bone metastases	BMP-6 +bonesialoprotein +thymidine phosphorylase	Tissue	IHC	[20]
	Osteoprotegerin	Serum	Immunofluorometric Assays	[64]
Determine early relapse androgen ablation therapy	Osteoprotegerin	Serum	Immunofluorometric Assays	[66]
Prediction of disease progression (circulating tumor cells).	PSMA/PSMA', PSCA	Tissue, Blood	Quantitative RT-PCR	[98,99],[25]
	$PCA3^{DD3}$	Blood		[100]
	Tβ15	Tissue	IHC	[50]
	Tβ15/protein-creatinine	Urine	Immunofluorometric Assays	[52,53]

BMP-6 and prostate cancer metastases, no research assays have been developed to test its diagnostic potential in body fluids (Table 3).

3.2. PSCA

Strong expression of prostate stem cell antigen (PSCA), a glycosylphosphatidylinositol-anchored cell-surface protein, was found in 72.7% of high-grade PIN, 83.4% of prostate cancers, 20% of BPH and 22.2% of low-grade PIN specimens [21,22]. PSCA expression was shown to correlate with Gleason score $\geq$7, invasion of the seminal vesicles, capsule involvement, and progression to an androgen-independent stage (Table 2) [21–23]. Recently PSCA expression was found in 87.2% of bone metastases, 66.7% of lymph node metastases, and 66.7% of liver metastases of prostate cancer [24].

The prognostic value of PSCA was shown by PSCA-specific RT-PCR analysis of peripheral blood of prostate cancer patients. The presence of PSCA mRNA in the peripheral blood of patients with extra prostatic disease was associated with a lower disease-progression free survival (Table 2)[25]. More studies are needed to support the clinical prognostic value of PSCA, using the more sensitive and reproducible quantitative RT-PCR assays.

4. Phase two biomarkers

4.1. AMACR

The diagnostic usefulness of α-Methylacyl-CoA racemase (AMACR) was shown in prostate needle biopsies where AMACR protein expression had 97% sensitivity and 100% specificity for prostate cancer detection (Table 2) [15,26]. In clinical practice, AMACR is used in combination with prostate cancer negative basal cell stains (p63, 34βE12, and cytokeratins 5 and 6) [27,28]. Currently, the accuracy and specificity of AMACR in the detection of prostate cancer in biopsy specimens is regarded as an improvement over the serum PSA-test [29].

Table 3
The current evaluation status of biomarkers

Biomarker	Substrate	Phase 1	Phase 2	Phase 3	Phase 4	Phase 5	Phase 6
BMP-6	Serum	yes	no	no	no	no	No
CRISP-3/SGP28	Serum	yes	ongoing	no	no	no	No
EPCA	Plasma	yes	ongoing	no	no	no	No
hK2	Serum	yes	yes	yes	no	no	No
OPG	Serum	yes	yes	no	no	Yes, Biomedica (Germany)	No
PSMA/PSMA'	Blood/Urine	yes	no	no	no	no	No
GSTP1	Urine	yes	ongoing	no	no	no	No
$PCA3^{DD3}$	Urine	yes	Yes, TRF-based QRT-PCR/$uPM3^{TM}$-test	ongoing	ongoing	End of 2005, Gen-Probe	After 2009 Gen-Probe
Tβ15	Serum/urine	yes	ongoing	no	no	no	No
Telomerase activity	Urine	yes	Yes (Roche Diagnostics)	no	no	Yes (Roche Diagnostics)	No
AMACR	serum/urine	yes	ongoing	no	no	no	No
PSCA	blood	yes	no	no	no	no	No

Phase 1: Exploratory study using home made first generation test.
Phase 2: Establishment of a reproducible assay; both inter- and intra assay variability should be assessed.
Phase 3: Retrospective-or prospective analysis of a biomarker using standardized/second generation test.
Phase 4: Prospective multi-centre evaluation of a biomarker.
Phase 5: Commercialization of a biomarker.
Phase 6: FDA approved.

There have been many efforts to develop a body fluid-based assay for AMACR. First, an AMACR activity assay used on extracts derived from prostate needle biopsy specimen had 92.3% sensitivity and 89.2% specificity for prostate cancer detection (Table 2) [30]. Second, an AMACR immune reactivity assay showed 71.8% specificity and 61.6% sensitivity in distinguishing the sera of prostate cancer patients from those of healthy controls, and could be used in combination with serum PSA to reduce the number of unnecessary biopsies (Table 2) [31]. Third, a small study indicated that AMACR-based quantitative real-time PCR analysis on urine samples obtained after prostate massage has the potential to exclude the patients with clinically insignificant disease when AMACR expression is normalized for PSA [32]. Fourth, Western blot analysis on urine samples obtained after prostate massage had a sensitivity of 100%, a specificity of 58%, a positive predictive value (PPV) of 72%, and a negative predictive value (NPV) of 88% for prostate cancer (Table 2).

The development of an ELISA for AMACR protein detection would be one step forward in the validation of this marker for prostate cancer (Table 3). Larger studies with long-term follow-up are needed to validate the clinical usefulness of this biomarker. Recently, Gen-Probe, global leader in development, manufacture and marketing of diagnostic nucleic acid tests, has licenced the rights from Corixa, a biopharmaceutical company, to develop molecular diagnostic tests for over 50 potential genetic markers, including AMACR.

4.2. CRISP-3/SGP28

Specific granule protein of 28 kDa (SGP28) was purified from human neutrophils and independently cloned from a human testis cDNA library [33,34]. It is currently known as cysteine-rich secretory protein 3 (CRISP-3). Human CRISP-3/SGP28 is a secretory protein [35,36]. A sensitive, quantitative CRISP-3/SGP28 ELISA assay showed its expression in human plasma, saliva, seminal plasma, and sweat. Combined with the fact that it has sequence homology with pathogenesis-related proteins, that are considered to play a role in plant anti-microbial defence, an antimicrobial role for CRISP-3/SGP28 has been suggested [37].

CRISP-3/SGP28 mRNA expression was found in pancreas, prostate, and salivary gland and at lower expression levels in colon, epididymis, ovary, and thymus [34]. The prostate tumor-associated over-expression of CRISP-3/SGP28 was determined and the up-regulation of CRISP-3/SGP28 mRNA was shown to be 20–20,000 fold in Gleason grade 3 prostate cancers (Table 2)[35,38]. Studies in mice indicated that CRISP-3/SGP28 is an androgen-dependent gene, although this has not been studied and confirmed in humans [39].

It has been suggested that like PSA, CRISP-3/SGP28 protein levels might be increased in plasma of prostate cancer patients. The developed quantitative ELISA-based assay can be used to study the diagnostic potential of CRISP-3/SGP28 in body fluids which makes CRISP-3/SGP28 a phase two biomarker (Table 3) [40].

4.3. EPCA

The expression of early prostate cancer antigen (EPCA), a prostate cancer-associated nuclear structural protein, was found both in cancer areas and adjacent normal tissue areas of prostate cancer tissue specimen but was absent in normal prostate tissue sections obtained from healthy donors and BPH samples [41]. IHC-analysis had 84% sensitivity and 85% specificity for prostate cancer. Furthermore, a positive EPCA staining in an initial prostate cancer negative biopsy may precede the diagnosis of prostate cancer as much as 5 years later. As such it could identify men at risk for prostate cancer at a much earlier time as they would have currently been diagnosed (Table 2) [42].

Using an EPCA-based ELISA, increased plasma EPCA levels had a sensitivity of 92% and a specificity of 94% for prostate cancer (Table 2). Patients with prostatitis were negative for EPCA and it appears that PIN or PIA lesions do not result in elevated detectable levels of EPCA in plasma as well [43].

EPCA is a fairly new biomarker in phase two of biomarker development (Table 3). Since only 12 prostate cancer patients were included in the previous study, larger studies are required, to demonstrate that the EPCA-based ELISA assay can distinguish prostate cancer patients from those with non-malignant prostatic diseases. Furthermore, the reproducibility of the assay needs to be assessed. The availability of such an assay enables well-controlled confirmatory studies to assess its real diagnostic value.

4.4. GSTP1

Recently, an overview has been given on the diagnostic applicability of the hypermethylation of the GSTP1 promoter which is the most commonly occurring epigenetic alteration in prostate cancer (Table 2) [15]. Hypermethylation of the GSTP1 promoter occurs in more than 90% of prostate tumors and has been significantly associated with Gleason score $\geq$7 as well as the presence of a single Gleason 4 and/or 5 grade [44,45]. Thus, hypermethylation of the GSTP1 promoter could also be a biomarker for prostate cancer aggressiveness.

For clinical purposes, differences in sensitivity between laboratories need to be overcome by standardization of the MSP technology. The drawbacks of the conventional qualitative MSP analysis are its labour intensiveness and its higher risk for contamination because of the frequent manipulation of PCR products. An improvement of MSP analysis has been a fluorescent-based real-time quantitative MSP analysis (QMPS), although this assay was less sensitive than the conventional MSP analysis [46,47]. Thus, an MSP analysis with both high sensitivity and specificity should be developed in a phase two study (Table 3). Positive GSTP1 hypermethylation results were observed for haematuria, which indicates that a limitation of this marker involves the contamination of the urine with erythrocyte GSTP1 [48]. Therefore, this standardized MSP assay should be used on urinary sediments to evaluate the real diagnostic value of GSTP1 hypermethylation in body fluids.

4.5. Thymosin β15

In the Dunning rat prostatic carcinoma model system, in which cell motility closely correlates with the metastatic phenotype, an actin-binding protein, thymosin beta 15 (Tβ15), was found to be up-regulated at both the mRNA and protein level. Tβ15 was found to be involved in the positive regulation of cell motility, which is a critical step in the metastatic pathway. The correlation of Tβ15 protein expression with metastatic potential was confirmed in mouse lung carcinoma cells and human breast carcinoma cells [49]. Tβ15 expression was also correlated with high-grade prostate cancers. Moreover, strong Tβ15 expression in Gleason 6/10 prostate cancers had a PPV of 86% for biochemical progression in patients who showed no metastases at initial presentation (Table 2). A weak Tβ15 staining had a NPV of 71% for biochemical progression [50,51].

Using a quantitative high-throughput ELISA assay it was determined that elevated Tβ15 protein concentrations in urine samples from patients prior to prostate cancer treatment precede prostate cancer recurrences (Table 2) [52,53]. The availability of a research assay makes multi-centre studies possible, although the reproducibility of the assay needs to be assessed in phase two studies (Table 3).

5. Phase three biomarkers

5.1. HK2

The potential of hK2 as a biomarker for prostate cancer has been discussed previously [15]. Highly sensitive hK2-specific immunoassays demonstrated the diagnostic applicability of the serum hK2 over free PSA ratio in the diagnostic ‘gray-zone’ to distinguish prostate cancer patients from men with BPH. Additionally, serum hK2 alone or in combination with total PSA and free PSA may improve the detection of extraprostatic or advanced disease (Tables 2 and 3) [54–57].

Using a quantitative RT-PCR method, hK2 protein expression was found to be increased in high grade PIN, prostate cancer and lymph node metastases compared with normal prostate tissue. Since the PSA

expression decreases in prostate cancer, the hK2/PSA mRNA ratio was increased in prostate cancer tissue compared with normal prostate tissue [58–60]. Further studies are needed to define whether the hK2/PSA ratio may distinguish the more aggressive tumors from the indolent ones, and whether there is a diagnostic implication for this test for the detection of prostate cancer in urinary sediments obtained after prostate massage (Table 2).

6. Phase 5 biomarkers

6.1. Osteoprotegerin

The most common site for prostate cancer metastases is the bone. In normal bone, the regulation of bone forming osteoblasts and bone degrading osteoclasts is balanced through osteoclastogenesis, which is regulated by the proteins, RANK, RANKL and osteoprotegerin (OPG) [61,62]. In bone metastases this balance is disturbed to facilitate the invasion and growth of tumor cells in the bone. The protein expression of RANKL and OPG were found to be significantly increased in cells of prostate cancer bone metastases compared with cells of primary prostate cancer or non osseous metastases indicating that serum levels of OPG might be increased in patients with prostate cancer bone metastases compared with serum levels of primary prostate cancer patients [63]. Using a sandwich-type ELISA, serum OPG levels were found to be significantly lower in patients with primary prostate cancer compared to men with BPH although this was not confirmed by an independent study [64,65]. Additionally, serum levels of OPG were found to be significantly higher in patients with advanced disease compared to patients at other stages of prostatic disease [65,66]. Serum OPG had 88% sensitivity and 93% specificity for the correct identification of patients with bone metastases (Table 2) [64]. Only a small improvement could be obtained in diagnostic accuracy when serum OPG was combined with other bone markers [67]. Elevation of serum OPG was not observed in bone metastases of other malignancies [68].

It has been suggested that increased serum OPG concentrations may be a marker of early relapse from androgen ablation therapies in patients with low serum PSA values (Table 2) [66]. However, when interpreting serum OPG levels it should be taken into account that serum concentrations of OPG are increased in case of vascular diseases or rheumatoid arthritis [69,70].

Although serum PSA is an excellent marker for the detection of disease recurrence it is not indicative of bone metastases except at levels greater than 20 ng/ml. Serum OPG seems a promising biomarker to indicate bone metastases and early relapse of disease. A commercially available ELISA for OPG measurement in serum (Biomedica, Germany) brings OPG to the fifth phase of biomarker development and facilitates phase three and phase four well-controlled studies to assess serum OPGs diagnostic/prognostic value (Table 3).

6.2. Telomerase

The diagnostic applicability of telomerase has been described previously [15]. High telomerase activity has been found in 90% of prostate cancers and was shown to be absent from normal prostate tissues. Using prostate fluid specimens obtained after prostate massage, telomerase activity had 90% sensitivity, 76% specificity, 87% PPV and 90% NPV for prostate cancer (Table 2) [71]. High telomerase activity was found after immuno-captivation of epithelial cells in the urine of 100% of prostate cancer patients and low to high telomerase activity was found in 30% of men with BPH [72].

Although telomerase activity has been successfully detected in prostate biopsy specimens, urine and prostatic fluid, its diagnostic value for early detection, staging or progression of disease needs still to be assessed. A telomerase polymerase chain reaction (PCR) ELISA kit has been developed and commercialized by Boehringer Mannheim (now part of Roche Diagnostics GmbH (Mannheim, Germany) (Table 3). This test has been used to determine telomerase activity in prostate needle biopsies and prostatic fluid specimen [73]. The availability of the commercially available TRAP assay enables the reproducibility of clinical results in multi-centre phase 3 and 4 studies.

6.3. $PCA3^{DD3}$

The diagnostic applicability of the most prostate cancer-specific gene, $PCA3^{DD3}$ has been described previously [15]. In phase two of biomarker development a second generation Time-resolved fluorescence-based quantitative RT-PCR assay was developed that has been used for the detection of $PCA3^{DD3}$ transcripts in urinary sediments obtained after DRE from a cohort of men who were indicated for prostate biopsies based on a total serum PSA value above 3 ng/ml (Table 2) [74]. Three independent studies showed that $PCA3^{DD3}$-based analysis had an average sensitivity of 72%, a specificity of 83%, and a NPV of 88% for prostate cancer detection using prostatic biopsies as a gold-standard for the presence of a tumor (Table 4) [74–76]. Several men included in the study published by Hessels et al., (2003) who had negative biopsies but a positive $PCA3^{DD3}$ -test were shown to have prostate cancer

Table 4
Performance of $PCA3^{DD3}$-based analysis for prostate cancer detection in urinary sediments after extended DRE

Sensitivity	Specificity	NPV	References
67%	83%	90%	[74]
82%	76%	87%	[75]
66%	89%	87%	[76]

upon repeated biopsies (unpublished data). Currently phase 3 and 4 studies are ongoing which are important to define that $PCA3^{DD3}$-based diagnostics can detect prostate cancers months or years before prostate cancer is diagnosed (Table 3).

DiagnoCure has developed the first-generation version of the $PCA3^{DD3}$-test, the $uPM3^{TM}$-test. Currently, Bostwick Laboratories (Richmond, USA) offer the $uPM3^{TM}$ test to their patients. Gen-Probe has the exclusive worldwide licence to the $PCA3^{DD3}$ technology and has successfully transferred the technology to its APTIMA® platform. APTIMA® uses transcription-mediated amplification (TMA), which is an RNA transcription amplification system using RNA polymerase and reverse transcriptase to drive the isothermal reaction. The risk of contamination is minimized since the technique is carried out in a single-tube format. When Gen-Probe introduces its APTIMA® version of the $PCA3^{DD3}$ test at the end of 2005, then multi-center studies using the validated $PCA3^{DD3}$ assay can provide the first basis for the molecular diagnostics in clinical urological practice.

7. Clinical application of biomarkers

Because there are no adequate therapeutic options for advanced prostate cancer, it is very important to detect prostate cancer at an early stage when it is potentially curable. Although the routine use of PSA testing has undoubtedly increased prostate cancer detection one of the main drawbacks has been the lack of specificity [77,78]. Serum PSA is an excellent marker for prostate disease and even modest elevations almost always reflect a disease or perturbation of the prostate gland including BPH and prostatitis. The lack of cancer specificity results in a high negative biopsy rate. Since the advent of frequent PSA testing over 15 years ago the specificity of PSA for cancer has declined due to the selection of a large number of men who have elevated PSA due to non-cancer mechanisms. However, PSA testing has been significantly refined and with tests for new PSA forms on the horizon, the way PSA can be used may be enhanced. Methods to enhance PSA specificity, which have gained popular use, include assessment of prostate size or PSA density, the rate of PSA increase with time (PSA velocity), age-specific or age-adjusted PSA 'cut-offs' and free PSA [77–80]. All of these methods, separately or in combination, have aided decisions for biopsy. The development and adoption of these clinical algorithms with an established test, illustrates the evolution of optimal use of new diagnostic tests and will no doubt apply to the new diagnostic paradigm of detecting cancer cells directly utilizing molecular probes. In particular, a very recent study of PSA velocity has revealed that men whose PSA increases by more than 2.0 ng/ml a year before diagnosis have a relatively high risk of death from prostate cancer even after radical prostatectomy [81].

The discovery of free PSA in 1990 launched the development of specific free PSA assays and a multitude of studies demonstrating the additional specificity for predicting positive biopsies with the ratio of free PSA to total PSA. The use of free PSA has been shown in some studies to reduce the number of negative biopsies by 25% [82,83]. Although total PSA increases with probability of cancer, free PSA shows an inverse relationship. The molecular nature of free PSA and the mechanism accounting for the correlation with benign disease was not understood for many years. It was known that native enzymatically-active PSA very rapidly forms essentially irreversible complexes with protease inhibitors in blood including alpha-2 macroglobulin and alpha-1 antichymotrypsin. It was therefore assumed and now has been demonstrated that free PSA must be different than the native enzymatically active form. It has now been shown that free PSA is actually three major molecular species [84]. BPSA or benign PSA is characterized by two distinct internal peptide bond cleavages and is therefore a degraded form of native PSA [85,86]. As expected BPSA is not enzymatically active and does not form complexes with protease inhibitors. Using a specific monoclonal antibody to BPSA, IHC studies have shown that BPSA is primarily located in the transition zone of the prostate with an increased expression in BPH nodules [86]. Another free PSA form, InPSA is not molecularly characterized but has also been shown to be strongly correlated with benign disease. These two forms, BPSA and InPSA represent the correlation of free PSA with benign disease. Recent studies have shown that BPSA is more closely correlated with prostate size and BPH than free PSA suggesting that this biomarker may be useful for monitoring therapy of BPH [87]. The third molecular form of free PSA is the precursor of mature PSA, proPSA and two major degraded forms of proPSA [88,89]. ProPSA is enzymatically inactive and

does not form complexes with protease inhibitors. Most interesting is the finding that monoclonal antibodies to proPSA and the two degraded forms has shown an increased immunostaining of these forms primarily in high grade PIN and prostate tumors [90]. Serum proPSA is correlated directly with cancer. The proportion of proPSA to total PSA has been shown to significantly improve the specificity for cancer compared to the %free PSA. In addition the %proPSA has shown a high correlation with aggressive tumors in the 2–10 ng/ml PSA range. These studies suggest that the inclusion of proPSA can increase specificity of prostate cancer and provide information regarding the prognostic course of a patient.

The many studies showing enhanced specificity and sensitivity as well as prognostic utility for PSA, PSA forms and hK2 strongly suggests that a biomarker panel will provide significantly more diagnostic information than any one biomarker [91]. It is likely that the future of immunodiagnostic tests for prostate cancer will trend toward the use of multiple markers using multiplex assay formats. These ''selective'' proteomic profiles will be quantitative and include algorithm analysis, possibly non-linear analysis like ANN [91]. Since the detection of cancer cells directly is not only expected to be more specific for cancer but also a completely independent analysis for cancer compared to the surrogate markers in serum it seems reasonable that both types of clinical diagnostic tests will be used effectively in the future to provide much more accurate diagnostic and therapeutic decisions.

To further improve the specificity prostate cancer diagnosis in the serum PSA 'gray-zone', the implementation of prostate cancer-specific markers is urgently needed. New markers have been provided and 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. It has become clear that many biomarkers discussed in this review are still in the first phases of biomarker development and that sensitive, reproducible research assays are needed for both biomarker testing in body fluids and for confirmation studies at different laboratories. Until now, only the diagnostic potential of GSTP1, $PCA3^{DD3}$, and telomerase have been studied in reducing the number of biopsies. Ironically, the invasive TRUS-guided prostate biopsies have been used as the 'gold' standard. Therefore, many patients who are currently regarded as being negative for prostate cancer may become cancer patients in the near future as was already shown in the $PCA3^{DD3}$ study. Follow-up data from clinical studies are important to improve outcome and management of prostate cancer.

The only biomarker that is currently progressing through the first five phases of evaluation is $PCA3^{DD3}$. Currently, $PCA3^{DD3}$-based tests are involved in a prospective screening study (phase 4). Marker commercialization (phase 5) will take place when Gen-Probe introduces the APTIMA® version of the $PCA3^{DD3}$ test (ASR) as an analyte specific reagent at the end of 2005. Then $PCA3^{DD3}$-based testing will be the first gene-based test that can be used as 'adjunct' to PSA testing (reflex-test). The next very important phase will be clinical trials leading to FDA approval. Only when the biomarker has delivered its diagnostic and prognostic promises, may it be introduced as a first line diagnostic adjunct in clinical practice.

Other markers are likely to follow $PCA3^{DD3}$ and may add additional value in the diagnosis of prostate cancer. It was shown that the combination of $PCA3^{DD3}$, Hepsin and PSMA was the best multivariate predictive model that distinguished 100% of the prostate cancer tissue specimens from the BPH tissue specimens [92]. Additionally, such a set of genes combined with a set of markers for disease aggressiveness could aid the urologist in his decision which patient would benefit from curative treatment and which patient would benefit from other therapeutic approaches. The close collaboration and communication between clinicians and researchers is essential in clinical testing of these markers to assess their real diagnostic potential and to evaluate the impact of these tests on the reduction of unnecessary biopsies and disease mortality.

8. Conclusions

Since prostate cancer is a heterogeneous disease, it becomes clear that a defined set of markers will become important in early diagnosis, monitoring and prognoses of prostate cancer. The list of potential markers will continue to grow and only through standardized sample collection, thorough, systematic evaluation and clinical testing of the potential biomarkers a greater insight into their true diagnostic potential emerges. The lack of standardization and reproducibility of research assays hamper the clinical investigation of these biomarkers in multi-centre studies. Therefore, it is essential that research groups use the proposed guidelines for biomarker development. It may take up to a decade to develop a biomarker going through all phases of development. The PSA test is a good example. The first PSA test from Hybritech (now Beckman Coulter) was released in 1985. It took an additional 9 years to obtain the FDA approval to use it in clinical practice for the detection of prostate cancer.

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CME questions

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1. For which of the following markers is NO FDA approved test available?
   A. PSA
   B. telomerase(hTERT)
   C. Osteoprotegerin(OPG)
   D. PCA3

2. Which gene/protein is overexpressed in prostate cancer?
   A. PSA
   B. hK2
   C. PCA3

D. all of the above

3. Which marker is indicative for bone turnover?
   A. PSA
   B. hK2
   C. osteoprotegerin
   D. PCA3

4. In prostate cancer the GSTpi gene is often
   A. overexpressed
   B. mutated
   C. methylated
   D. glycosylated