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Original Article

Risk stratification of prostate cancer patients based on EPS-urine zinc content

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Abstract: The early detection of prostate cancer is a life-saving event in patients harboring potentially aggressive disease. With the development of malignancy there is a dramatic reduction in the zinc content of prostate tissue associated with the inability of cancer cells to accumulate the ion. In the current study, we utilized endogenous zinc as a diagnostic biomarker for prostate cancer. We employed a novel fluorescent sensor for mobile zinc (ZPP1) to measure the concentration of zinc in thirty-nine patient samples of expressed prostatic secretion (EPS) in urine. We estimated the probability of classifying a subject as benign, low-risk, or high-risk as functions of the diagnostic test results using a non-informative prior Bayesian approach. Permutation tests and other non-parametric tests were also used. We demonstrated a significant trend in zinc score with disease and with disease risk (P = 0.03), and lack of a significant correlation between zinc score and PSA. We also showed that the proposed diagnostic is potentially superior to PSA for detecting high-risk disease. Considering that risk stratification represents an important unmet need, our method should advance the field of prostate cancer diagnostics and treatment planning.

Keywords: Diagnostics, prostate, cancer, zinc, risk-stratification

Introduction

Prostate cancer is the second leading cause of cancer death in men, exceeded only by lung cancer [1]. According to the American Cancer Society, it accounts for about 13 per cent of male cancer-related deaths. The five-year disease-specific survival rate for localized cancer is 100%. By contrast, metastatic prostate cancer is not curable and has an overall five-year survival of just 33%. Life expectancy can be as low as 13 months, even in the presence of androgen-deprivation therapy [2]. The significance of accurate diagnosis and intervention is especially pronounced when considering younger men. A comprehensive recent study demonstrated that men under 50 more commonly had organ-confined disease and a pathologic Gleason score ≤ 6, implying that early diagnosis of disease is associated with more favorable outcomes and that population-based screening at younger ages could potentially lead to improved survival [3].

There is, however, an overall lack of reliable diagnostic and staging tools for prostate cancer. The current clinical diagnosis and staging of a prostate cancer relies on four core parameters: digital rectal examination (DRE), serum prostate-specific antigen (PSA), biopsy, and imaging [4]. Among them, the PSA test represents an important initial screening tool. Although many men with prostate cancer have an elevated PSA concentration (greater than 4.0 ng/mL), a high level does not necessarily mean that there is cancer. A false positive rate as high as 70% has been reported [5]. Conversely, 15% of men with prostate cancer will not have an elevated PSA measurement. In fact, close to 7% of men with a PSA of 0.5 ng/mL or lower will have prostate cancer and, of those, 12% will have high-grade disease [6]. Improvements based on the PSA test, such as the measurement of PSA velocity and free PSA, are also used clinically. However, neither PSA velocity [7] nor free PSA have consistently been

Table 1. Patient Characteristics

disease state	stage	gleason score	tumor volume (cm^3)	Risk/ disease	psa
Benign	na	na	0	PIN	0
	na	na	0	BPH	0
	na	na	0	BPH	4.6
	na	na	0	BPH	0
	na	na	0	BPH	0
	na	na	0	BPH	4.6
	na	na	0	N	1.8
	na	na	0	N	0
	na	na	0	PIN	3.7
	na	na	0	BPH	3.1
	na	na	0	N	6.8
	na	na	0	N	4.2
	na	na	0	N	9.4
	na	na	0	N	10.5
	na	na	0	PIN	23.4
Low-risk	T2c	3+3	0	L	5.7
	CT2ANXMX	3+3	0	L	4.2
	CT2ANXMX	3+3	0	L	2.5
	CT1CNXMX	3+3	0	L	1.8
	CT1CNXMX	< 6	0	L	2.6
	CT1CNXMX	3+3	0	L	3.5
	CT2ANXMX	3+3	0	L	7.93
	CT1CNXMX	3+3	0	L	4.52
	T2c	3+3	0	L	3.4
	T2c	3+4	0	L	2.3
	T2c	3+3	0	L	9.8
	CT1CNXMX	3+3	0	L	11.1
Intermediate/High-risk	Unknown	3+4	0	I	8.8
	T2c	4+3	15	I	2.6
	T2c	4+3	0	I	4.9
	T2a	4+4	2	H	6.1
	T2c	4+4	7	H	0
	T3a	3+4	25	H	0
	CT2ANXMX	4+4	0	H	26.6
	CT1CNXMX	4+5	0	H	0.2
	CT3BNXMX	4+3	0	H	0
	CT3BNXMX	4+3	0	H	0
	CT2BNXMX	4+5	0	H	0.72
	CT2CNXMX	5+5	0	H	65.7

shown to enhance the specificity of prostate cancer detection over PSA level alone [8].

Importantly, PSA is not useful for predicting disease risk among patients diagnosed with prostate cancer. Among patients in the placebo arm of the Prostate Cancer Prevention Trial, (N = 5519), 4.7% were diagnosed with prostate cancer, and 141/259 = 44% of these had a PSA less than 4.0 [6, 9]. Keeping in mind that prostate cancer is very slow growing and in many cases men harboring the disease can be managed successfully with watchful waiting, it is imperative to develop tools for risk stratification [10]. The lack of reliable noninvasive or minimally invasive methods for risk prediction means that many men will either undergo unnecessary invasive and emotionally taxing treatment or remain undiagnosed.

Therefore, a true staging marker of prostate cancer risk is urgently needed. Indeed, over half a century of research has identified zinc as an excellent candidate biomarker. The healthy prostate contains the highest concentrations of mobile zinc of all soft tissues. These levels decrease dramatically during the development of prostate cancer, even at an early stage [11]. Reportedly, the zinc concentration in the malignant peripheral prostate, which is the main region of cancer development, is reduced 6-fold compared to the normal peripheral prostate (500 vs. 3000 nmols/g). This difference is even more dramatic in prostate fluid (1000 vs. 9000 nmols/g) [12].

The importance of zinc as a risk stratification biomarker was further confirmed by computer modeling of histological sections from patient biopsies. According to these studies zinc concentrations in prostatic tissues have the potential for risk prediction and differential diagnosis in agreement with tumor grade obtained by biopsy [13, 14].

Table 2. Clinical Definitions

Risk	Definition
Benign	Normal: prostate volume < 30 cc, normal voiding history, PSA 2.5-10, negative prostate biopsy
	BPH: prostate volume > 30 cc, bladder outlet obstructive symptoms, index > 14, biopsy negative; PSA 2.5-10
Low-Risk	PSA < 10, Gleason 3+3 or less, clinical stage T2a or less, < 30% biopsies core positive
Intermediate-Risk	Gleason 4+3 or 3+4, or stage T2b or T2c, or PSA 10-20, 30-50% biopsies core positive
High-Risk	PSA > 10, Gleason score 4+4 or higher, > 50% biopsies core positive, any T stage with the Gleason score noted

Therefore, in this study we focused our attention on zinc as a prostate cancer biomarker. We developed an assay that allows us to quantify biological mobile zinc. The assay uses a new zinc sensor (ZPP1) with a unique turn-on, biphasic response to zinc [15, 16]. This special property of ZPP1 opens up the excellent opportunity to accurately quantify zinc in prostate cancer samples, using simple titration of the sensor [15, 16]. More importantly, the developed assay showed the possibility for risk assessment in cancer patients. The rapid development, validation, and introduction of this technology into the clinic has the potential to significantly increase the sensitivity and specificity of prostate cancer detection, as well as to provide an additional tool for disease staging.

Materials and methods

Chemical reagents

The cell membrane-permeable fluorescent Zn^{2+} sensor ZPP1 was prepared and characterized as previously described [15].

Patients

Patient characteristics are reported in **Table 1**. All samples were procured using protocols approved by the EVMS Institutional Review Board, and all applicable NIH guidelines and HIPAA regulations were followed. No personal information or identifiers were available to the laboratory investigators other than diagnosis, age, and lab results. All experiments were performed with approval by the institutional review board of the Massachusetts General Hospital and in accordance with an assurance filed with and approved by the U.S. Department of Health and Human Services.

Clinical samples

The expressed prostate secretion fluids were obtained following gentle prostate massage during digital rectal examination prior to biopsy. The massage consists of three strokes on each side of the median sulcus of the prostate. This procedure forces the expressed fluid from the glandular network of the prostate directly into the urethra. Urine (10-20 ml) containing the EPS was then collected from each individual and stored on ice. At the bio-repository, 9 ml of each sample were centrifuged to remove the cell pellet/particulate, and tubes of 0.5 ml, 1 ml (X4), and 4.5 ml were stored at -80°C. Cell pellets/sediment was also stored.

It should be emphasized that all of the EPS urine samples were obtained from men at the clinic just prior to their biopsy procedure. Hence, even those individuals with no evidence of cancer, and those with biopsy confirmed BPH, generally will have serum PSA values in the 2.5-10 ng/ml ranges. The EPS samples are initially classified using the currently accepted risk stratification system and include the results of a biopsy with a minimum of 12 cores. These classifications are presented in **Table 2**.

Measurement of EPS-urine zinc content ("ZPP1 test")

Measurement of zinc concentration using ZPP1 titration: Titrations were performed as previously described [15, 17]. Briefly, EPS urine samples were centrifuged at 2500 rpm for 5 min and diluted (1:4) in HEPES/KCl buffer (25 mmol/L HEPES and 100 mmol/L KCl; pH 7.0).

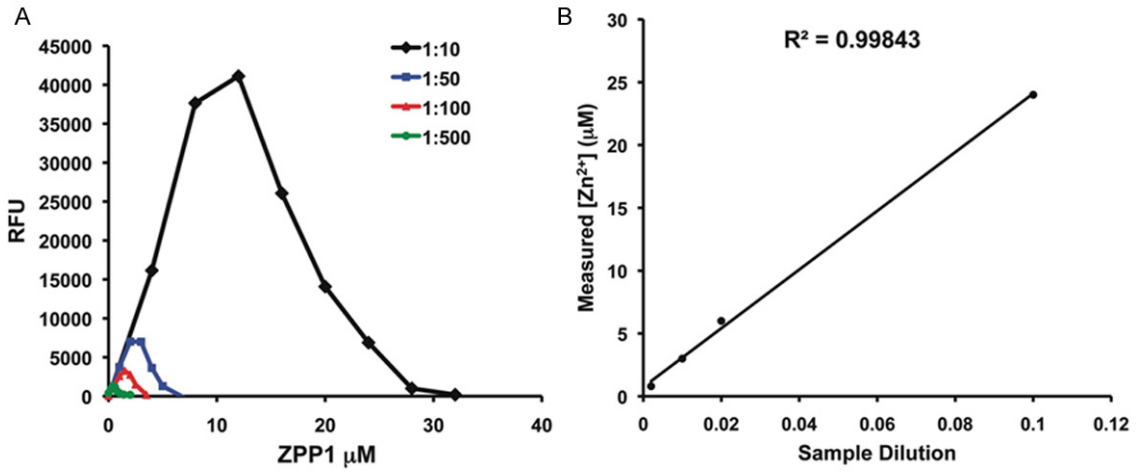

Figure 1. Zinc measurement by ZPP1 titration in serial dilutions of an EPS-urine sample from a non-cancer patient. A. Representative titration plot. B. Correlation between sample dilution and the zinc concentration measured using the ZPP1 assay over the range of tested dilutions. There was a high linear correlation (R^2 = 0.998) between sample dilution and the zinc concentration measured using the ZPP1 assay.

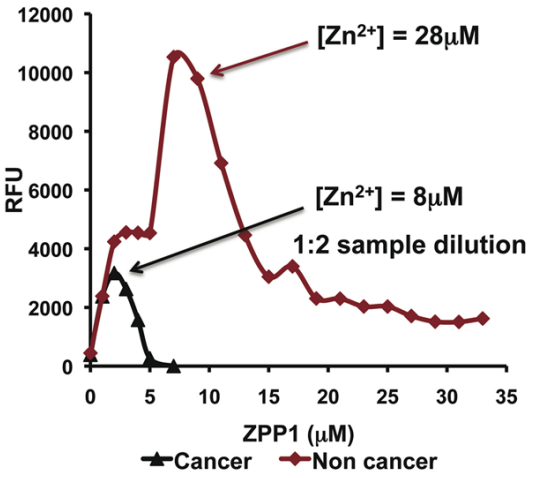

Figure 2. Quantification of zinc concentration by ZPP1 titration in EPS urine from a pool of non-cancer and cancer patients. The measured zinc concentration was 3.5-fold higher in the non-cancer group.

For fluorescence analysis of zinc concentration, 0.1 ml diluted EPS urine samples were added to 96-well plates. ZPP1 was titrated into the sample to achieve stepwise increments in ZPP1 concentration. At each step, the fluorescence was measured (excitation, 505 nm; emission, 532 nm) using a SpectraMax M2 fluorescence spectrophotometer (Molecular Devices). For each measurement, the fluorescence of buffer containing and equivalent amount of ZPP1 alone was subtracted from that of the sample. Because of the chemical properties of ZPP1, the titration curve gives a maximum when its concentration is exactly half that of the mobile zinc in the test solution. The zinc concentration can then be calculated according to the formula: $[Zn^{2+}] = 2[ZPP1]_{max}$

In accord with the literature [15], initial validation experiments in buffer confirmed that the ZPP1 concentration at the peak fluorescence equals half of the zinc concentration in the sample and that the measurements were linear over a range of 0.02-5 mM $[Zn^{2+}]$.

Determination of zinc score: The zinc scores were obtained by technicians blinded to the subjects' disease category. To determine zinc score, the EPS-urine zinc content (μM) of each patient was multiplied by creatinine concentration (μg/dL), in order to account for differences in the prostatic fluid fraction of the samples.

To measure the creatinine level in EPS urine, a commercially available kit from Cayman (Ann Arbor, MI) was used.

The zinc score was not obtained for one subject (PSA = 65, high-risk), for reasons independent of the subject's disease category. This subject was omitted from the analysis below.

Statistical analysis

Data analysis was performed using the R package for statistical analysis and graphics [18].

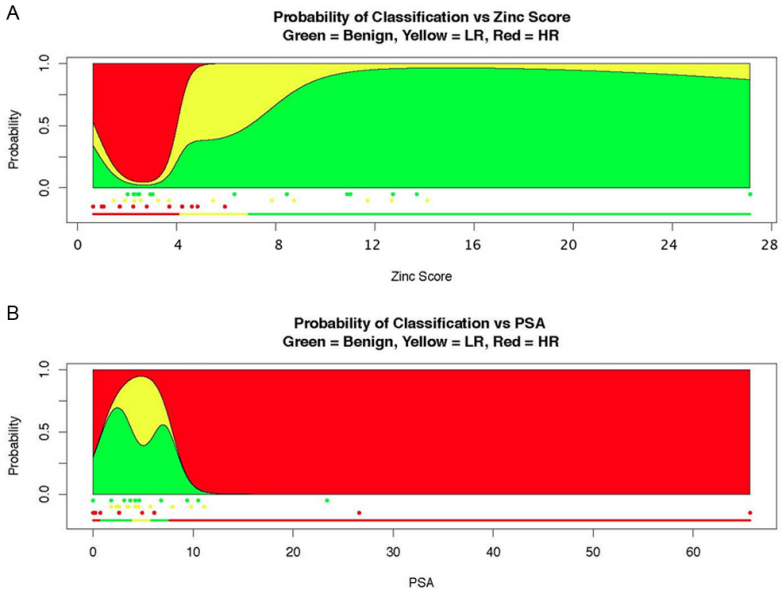

Figure 3. Data analysis. This figure illustrates the posterior probability of disease category as functions of zinc score (A) and PSA (B). The quantities on the horizontal axes are assumed to be normally distributed given the category (i.e., Benign, Low-Risk, or High-Risk). The priors for the normal means and standard deviations are constant and reciprocal, respectively. The prior for the category probabilities is the proportions of the data in each category. The colored dots are the data values in each category. The colored bars indicate which category has the highest probability as function of the zinc score or PSA.

The diagnostic biomarker (PSA or zinc score) values were assumed to be normally distributed by disease category (Benign, Low-Risk, High-Risk), with means and standard deviations that depended on the category. The proportions of subjects in each category were used as the prior probability of category classification. For the category means and standard deviations, we used the usual non-informative priors, i.e., proportional to a constant and to the reciprocal standard deviation, respectively. Since under these assumptions the posterior distributions of the biomarker value given the category will have the usual t-distributions for simple random samples [19], it is straight forward to compute numerically the posterior probabilities of category classification as functions of the biomarker values.

We had strong a-priori reasons to expect a monotone ordering of the three group mean zinc scores, with non-cancer highest, high-risk lowest, and low-risk in between. We constructed a nonparametric test of this trend as follows. First, we determined a non-parametric *p*-value for the maximum absolute difference in the three means from the appropriate permutation distribution. Next, we noted that conditional on this maximum absolute difference, the probability that the second largest mean is the low-risk mean is 1/3. So we divided the permutation *p*-value by three to get the final result.

We also tested the significance of the correlations between zinc score and PSA combined and within-group using the appropriate permutation distributions of the Pearson correlation and compared the performance of PSA and the proposed zinc score as diagnostic tests using McNemar's test [20].

Results

Sensitivity of the ZPP1 assay to zinc content and disease state using human EPS-urine

Before assessing the diagnostic value of the ZPP1 test for prostate cancer, we first determined the sensitivity of the assay to zinc concentration in EPS-urine. We prepared serial dilutions of an EPS-urine sample from a non-cancer patient and measured their zinc concentrations by ZPP1 titration (**Figure 1A**). We found a high linear correlation (R^2 = 0.998) between sample dilution and the zinc concentration measured using the ZPP1 assay over the entire range of tested dilutions ($[Zn^{2+}]$ = 800 nM - 25 mM; **Figure 1B**). This result suggested that our assay could be used to accurately quantify sub-micromolar zinc concentrations in EPS-urine. Furthermore, since the expected measurements of EPS-urine zinc content in the entire patient population fall within the tested range, we felt confident that our method could be used to probe for differences as a function of disease state (**Figure 1B**).

Next, we sought to determine whether the ZPP1 test could differentiate between non-cancer and cancer based on the zinc content of EPS-urine. We pooled EPS-urine samples from non-cancer and cancer patients (n = 25 in each pool). The non-cancer pool included samples

Table 3. Predictions vs. actual disease state for zinc score and PSA. Note that the 5 malignancies misclassified as benign by the zinc score were all low-risk. Of the 13 malignancies misclassified as benign by PSA, 7 were high-grade

Disease State	Prediction			
	Score Zinc		PSA	
	Benign	Malignant	Benign	Malignant
Benign	6	9	8	7
Malignant	5	18	13	10

Table 4. Results of using zinc score to classify subjects, with thresholds of ≤ 7 for malignancy, and ≤ 4 for high-risk malignancy

Disease State	Prediction		
	Benign	Low-risk	High-risk
Benign	6	1	8
Low-risk	5	1	6
High-risk	0	4	7

from BPH and normal subjects, whereas the cancer pool included samples representative of the entire spectrum of the disease. Using the ZPP1 test, we detected a 3.5-fold reduction in the zinc content of EPS-urine from cancer patients relative to non-cancer controls (**Figure 2**).

These results suggested that the ZPP1 test could report on the zinc content of human EPS-urine and, on this basis, has the capacity to differentiate between cancer and non-cancer. Having validated the assay in these studies, we performed risk stratification experiments in a limited number of patient samples.

Application of the ZPP1 test for prostate cancer diagnosis and staging

To investigate the value of the ZPP1 test as a clinical diagnostic and staging tool, we collected EPS urine samples from non-cancer/benign (n = 15), low-risk cancer (n = 12), and high-risk cancer (n = 12) patients, as determined by prostate biopsy. Each sample was assigned a zinc score based on the concentration of zinc obtained from the ZPP1 assay and taking into account the creatinine content of the sample. The mean zinc scores were 7.5, 6.4 and 2.8, for non-cancer, low-risk and high-risk, respectively. A test of the hypothesis that the group means were different revealed no significance ($F(2,35) = 2.4$, $P = 0.11$). However, there was strong a-priori reason to expect the zinc score to decrease monotonically with disease severity. The range of these values is 4.7 ($P = 0.09$, permutation test). However, in addition to a range of this magnitude, we observed the central mean to be low-risk, as predicted. The probability of this is 1/3, so the final *p*-value for the hypothesized trend in group-mean zinc scores is 0.03. The zinc score was not significantly correlated with PSA, either overall or for any of the three groups considered separately (permutation tests).

In order to illustrate the potential of zinc score as a predictor of disease category, we considered the following statistical model. Assume that zinc scores are normally distributed given the category, with means and standard deviations that depend on the category. For a Bayesian model with the usual non-informative priors on the normal distribution parameters, it is clear that the posterior distributions of the mean zinc scores given category have t-distributions [19]. We assume here that the prior probability of a zinc score coming from a subject in a particular disease category is equal to the proportion of subjects in our sample observed to be in this category. With these assumptions, it is straightforward to calculate the posterior probability of category assignment as a function of zinc score, and also as a function of PSA. The results of these calculations are given in **Figure 3**. From this Figure, it appears that the usual threshold of 4.0 for PSA seems to be reasonable. For the zinc score, a threshold of ≤ 7 to separate malignant from benign, and a second threshold of ≥ 4 to separate low-grade from high-grade appear to be reasonable.

We compared zinc score and PSA for distinguishing a benign condition from malignant disease (**Table 3**). The error fraction was less for the zinc score (14/38) than for the PSA (20/38), although not significantly so ($P = 0.30$, McNemar's test). However, there is a much greater difference in the diagnostic test performance than these comparable overall error rates suggest. The PSA test makes almost twice as many (13 vs. seven) false-negative predictions as false-positives, whereas the zinc score has only about half as many false-nega-

tives as false-positives (five vs. nine). The false-negative cases from the PSA test divide nearly evenly between high- and low-risk (seven high-risk, six low-risk). From **Figure 3**, it is apparent that PSA is not useful for separating high-risk from low-risk malignancies. On the other hand, the five false-negative cases from the zinc score test are all low-risk. It is also notable that eight of the nine false-positives for the zinc score test are classed as high risk and the total number of false-positive readings represented 37.5% of the combined low- and high-risk cancer-diagnosed patients (**Table 4**). The definition of these samples as false positives, however, can be questioned, taking into account that risk assessment in our study was based on biopsy- and not post-prostatectomy samples. Because, in 30-40% of the cases [21-23], Gleason grades, and therefore risk, are upgraded after prostatectomy, the 40% incidence of "false positives" in our data set could actually represent under-diagnosed true positives.

Discussion

The ambiguity associated with the current diagnostic tests for prostate cancer forces many men to either undergo unnecessary invasive and emotionally taxing treatment or remain undiagnosed. This dilemma has caused many clinicians and scientists to challenge the notion that testing for prostate cancer is warranted due to the slow-growing nature of the malignancy and its reduced influence on the overall survival of older men with a shorter life expectancy. However, recent trials have affirmed the life-saving value of early diagnosis, especially in younger men [3, 24]. Because, according to the American Cancer Society, 1 in 35 men in the U.S. will die of prostate cancer, there should be no debate about the need for an effective and reliable diagnostic tool as a facilitator of successful therapy.

In response to this need, we have developed a novel zinc-based diagnostic test for prostate cancer, which is strictly quantitative and predictive of disease aggressiveness.

Our approach centers around an established prostate cancer biomarker, which, despite having been known to the scientific community for over 50 years, has not fulfilled its promise in a clinical setting, owing to the absence of appropriate clinically relevant tools. This biomarker is most attractive because, whereas the concentration of mobile reactive zinc in the healthy prostate is the highest of all soft tissues in the body, there is a dramatic reduction in prostatic zinc content with the development of malignancy. This effect is specific to prostate cancer and can resolve the differential diagnosis between cancer and benign conditions, such as BPH [25] and prostatitis [26].

The presented diagnostic approach takes advantage of a recently described zinc-sensing fluorescent probe [15] and a simple high-throughput method for measuring zinc concentration in biological samples [15, 17]. This test provides information of unprecedented value, since none of the current diagnostic methods can accurately and noninvasively define disease aggressiveness. In spite of a seemingly high false-positive rate of the assay, we emphasize that 89% of these false positives were classified by our test as high risk. In addition, as mentioned above, 30-40% of all cases are reclassified based on postprostatectomy histopathology to a higher grade [21-23]. There is a high probability that our test could identify these under-diagnosed cases. A further advantage that cannot be underestimated is the ease, rapidity, and convenience with which this test can be performed. In addition, obtaining samples from the patients can be easily incorporated into the routine screening protocol, as a supplement to the DRE procedure. These advantages underscore the likelihood that the test will find clinical application as an addition to the established screening tools. In its totality, this new diagnostic paradigm could adequately address the critical need of risk prediction in the management of prostate cancer.

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