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ORIGINAL ARTICLE

Prostate Disease

Prospective validation of %p2PSA and the Prostate Health Index, in prostate cancer detection in initial prostate biopsies of Asian men, with total PSA 4–10 ng ml⁻¹

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Despite its widespread use for prostate cancer screening, low specificity makes PSA a suboptimal biomarker, especially in the diagnostic “gray zone” of 4–10 ng ml⁻¹. False-positives lead to unnecessary biopsies with attendant morbidities. This is the first prospective validation study of %p2PSA and the Prostate Health Index (PHI) in Asian men presenting with a total PSA between 4.0 and 10 ng ml⁻¹. We studied 157 Asian men between 50 and 75 years old, with normal per rectal prostate examinations, undergoing their first prostate biopsy, using a standardized biopsy protocol, for PSA levels of 4–10 ng ml⁻¹. Thirty (19.1%) were found to have prostate cancer on biopsy. Statistically significant differences between patients with and without prostate cancer were found for total PSA, p2PSA, %p2PSA, and PHI. The areas under the curve of the receiver operating characteristic curve for total PSA, %fPSA, %p2PSA, and PHI were 0.479, 0.420, 0.695, and 0.794, respectively. PHI predicts prostatic biopsies results best. At a sensitivity of 90%, the specificity (95% CI) of PHI was 58.3%, more than triple the specificity of total PSA at 17.3%, potentially avoiding 77 (49%) unnecessary biopsies. Similar to studies in mainly Caucasian populations, we have prospectively shown that %p2PSA and PHI greatly outperform total and free to total PSA ratio, in the detection of prostate cancer at first biopsy. Higher PHI levels also correspond to increasing the risk of detecting GS \geq 7 cancers. We have validated the use of PHI to aid decision-making regarding prostate biopsies in Asian men with serum PSA between 4 and 10 ng ml⁻¹.

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INTRODUCTION

Globally, prostate cancer (PCa) is the 2nd most frequently diagnosed cancer in men. Incidence rates vary by more than 25-fold worldwide and are highest in Australia/New Zealand, Northern America, Northern and Western Europe, and some Caribbean nations, and lowest in Asia.¹ Although the incidence is lower in Asia, PCa mortality rates are relatively higher. The 5-year survival rates for prostate cancer in Asia are generally much lower than the >95% rate reported in the United States, ranging from 40% in Mongolia to 87% in Japan.² Thus, although the incidence of PCa is lower in Asia, its early detection remains an important public health issue.

The advent of the PSA test has led to a dramatic reduction in the proportion of men diagnosed with metastatic disease and PCa death rates. Unfortunately, its low specificity has caused the problems of unnecessary biopsies with its associated morbidities, and overdiagnosis and overtreatment of indolent cancers.

Several approaches have been proposed to address the limitations of PSA, including the measurement of PSA molecular isoforms of free

PSA (fPSA). Free PSA (fPSA) comprises proPSAs (pPSAs), benign PSA (BPSA), and intact PSA. Four different proPSA isoforms exist in serum, named as [-2]proPSA, [-4]proPSA, [-5]proPSA and [-7]proPSA, based on the length of the pro-leader peptide sequences, i.e., seven, five, four or two amino acids. The [-2]proPSA (p2PSA) is the most cancer-specific form of all, being preferentially concentrated in cancerous tissue on histochemical staining and³ significantly increased in serum of men with PCa.⁴

The Prostate Health Index (PHI) developed by Beckman Coulter, Inc., in partnership with the NCI Early Detection Research Network was approved by the FDA in 2012. This is a mathematical formula of three biomarkers: $-(p2PSA/fPSA) \times \sqrt{PSA}$. By use of this calculation, the clinician will be able to see each individual result as well as make a potentially better informed recommendation to the patient.

This is the first prospective evaluation of %p2PSA and PHI in a cohort of Asian men undergoing their first biopsy within the diagnostic gray zone of total PSA 4–10 ng ml⁻¹.

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MATERIALS AND METHODS

Study design

The current study is a two institutional observational prospective cohort study, carried out from August 2012 to October 2014.

Study population

The study included consecutive men undergoing their first outpatient prostate biopsy for suspected PCa. Inclusion criteria were the following: patients 50–75 years of age with normal digital rectal examination (DRE) in a total PSA range of 4–10 ng ml⁻¹.

Exclusion criteria were as follows: First, patients with bacterial acute prostatitis or untreated urinary tract infection. Second, patients subjected to previous endoscopic surgery of the prostate. Third, patients with prior history of prostate cancer or other urogenital cancers. Finally, patients subjected to previous prostate biopsy or patients being treated with dutasteride or finasteride. Patients who were under any of the conditions above were excluded.

Study end points

The primary end point was to evaluate the sensitivity, specificity, and diagnostic accuracy of p2PSA, %p2PSA and PHI (index tests) in determining the presence of PCa at prostate biopsy in comparison to tPSA, fPSA, and percentage of free to total PSA ratio (standard tests). The number of prostate biopsies that could be spared if index tests were used in the prostate biopsy decision path was calculated.

Methods

Prior to prostate biopsy, blood was drawn to measure the prebiopsy total PSA (tPSA), fPSA, and p2PSA levels. Patients then underwent transrectal ultrasound-guided prostate biopsies according to a standardized extended scheme: at least 12 biopsy cores were taken, with additional cores taken if the performing clinician felt more cores were needed for adequate sampling. The specimens were processed and evaluated at each center by experienced genitourinary pathologists, who were blinded to the test results. PCa was identified and graded according to the 2005 consensus conference of the International Society of Urological Pathology⁵ definitions. Patients diagnosed with high-grade intraepithelial neoplasia or suspicious lesions but not adenocarcinoma (atypical small acinar proliferation of prostate) according to the contemporary diagnostic criteria were not considered positive for the outcome of interest.

All laboratory analyses (free PSA, total PSA and p2PSA) were performed on serum samples (collected into Becton Dickinson SST II tubes) which were centrifuged at 2000 g for 4 minutes within 3 h of collection and subsequently stored at -70°C until analysis. Testing was performed on a Beckman Coulter Dxi-800 immunoassay analyzer using manufacturer-supplied reagents, calibrators and controls in two analytical batches. All assays used chemiluminescent immunoassay technology with Hybritech PSA standardization. The Beckman Coulter Prostate Health Index (PHI) was calculated by the formula: $PHI = (p2PSA/fPSA) \times \sqrt{tPSA}$.

Statistical analysis

We summarized the distribution of continuous normally distributed demographic and clinical characteristics of patients using mean and standard deviation, and the differences in mean between the prostate cancer and noncancer groups were compared using the independent sample *t*-test. For skewed continuous variables, the median and range were used for summarizing the distribution, with comparisons between groups made via the Mann–Whitney test. For categorical variables, the χ^2 test was used for comparing differences in proportions.

The area under the receiver operating characteristic (ROC) curve was estimated for the various PSA derivatives and compared by assuming total PSA as the gold standard. The *P* value thus obtained were Bonferroni corrected. The specificity of the various PSA derivatives were also estimated at prespecified sensitivity of 90%.

All statistical analyses were carried out using STATA version 13.0 (StataCorp LP, College Station, TX, USA), assuming a two-sided test with a 5% level of significance.

The Institutional Review Board of the National Healthcare Group approved this study. Reagents for the study were supplied gratis by Beckman Coulter, but the sponsor had no input toward study design and were not given access to the study results nor had any input in the analysis of the results.

RESULTS

The patient characteristics of the 157 men recruited, and values of the various PSA parameters for this cohort are described in **Table 1**. The positive biopsy rate was 19.1% (30/157). Among the 30 patients diagnosed with prostate cancer, 11 (36.7%) had Gleason score 6 disease, 17 (56.7%) had Gleason score 7 disease, and 2 (6.7%) had Gleason 8–10 disease.

Between the positive and negative biopsy groups, there was no statistically significant difference in the mean total PSA ($P = 0.334$), median free PSA ($P = 0.148$), and the free to total PSA ratio ($P = 0.172$). On the other hand, there was a statistical significant difference for the median p2PSA ($P = 0.001$), median %p2PSA ($P = 0.001$) and PHI ($P < 0.001$) between the two groups.

The area under the ROC of tPSA, free: total PSA ratio, p2PSA, %p2PSA and PHI were 0.4787, 0.4197, 0.6950, 0.6895, 0.7937, respectively (**Table 2** and **Figure 1**). Of the various parameters, the PHI showed the best performance in predicting the results of the initial biopsy in this cohort.

To assess the performance of the various parameters further, we analyzed the data at a preset sensitivity level of 90% (**Table 3**). The PHI had the best specificity of 58.27% (95% confidence interval: 49.19–66.95) compared to 17.32% for total PSA. At a cut-off for biopsy at PHI level 26.75, 77 patients or 49.0% of this cohort could have avoided undergoing a biopsy. Of the three cancers that would have been missed at this cut-off, two were GS 3 + 3 and one was GS 4 + 3.

Looking at the detection of GS ≥ 7 cancer at 90% sensitivity, we found that at the same PHI threshold of 26.75, there was a similar specificity of 55.1%. We also looked at how the PHI test would perform using the manufacturer's banding of PHI levels (**Tables 4** and **5**).

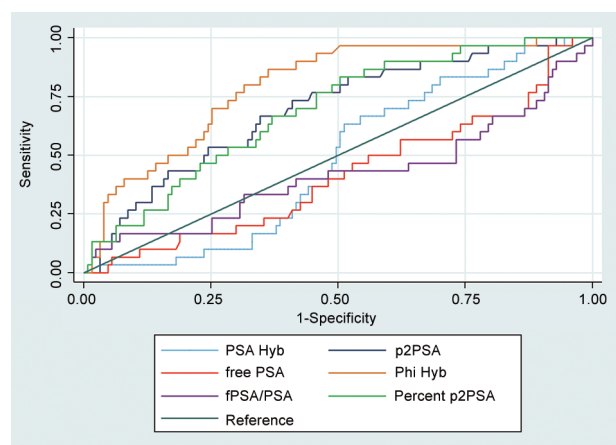


Figure 1: Receiver operating characteristic (ROC) curves comparing various PSA derivatives.

Table 1: Demographic and clinical characteristics of study subjects

Risk factor	Total (%) (n=157)	Cancer (%) (n=30)	No cancer (%) (n=127)	P
Mean age, years (s.d.)	65.4 (6.46)	68.3 (6.28)	64.7 (6.33)	0.005
Ethnicity (%)				
Chinese	146 (93.0)	25 (83.3)	121 (95.3)	0.033
Malay	5 (3.2)	3 (10.0)	2 (1.6)	
Indian	4 (2.6)	2 (6.7)	2 (1.6)	
Other	2 (1.3)	0 (0.0)	2 (1.6)	
Mean total PSA (s.d.)	6.71 (2.69)	6.28 (1.77)	6.81 (2.86)	0.334
Median free PSA (range)	1.10 (0.21–7.84)	0.95 (0.42–2.64)	1.1 (0.21–7.84)	0.148
Median free:total PSA ratio (range)	0.17 (0.06–0.51)	0.14 (0.06–0.41)	0.17 (0.07–0.51)	0.172
Median p2PSA (range)	11.5 (3.0–61.3)	15.0 (5.3–32.1)	10.9 (3.0–61.3)	0.001
Median %p2PSA (range)	1.80 (0.34–8.69)	2.36 (1.06–7.95)	1.60 (0.33–8.69)	0.001
Median PHI (range)	26.8 (6.5–93.7)	37.5 (14.1–90.0)	24.0 (6.5–93.7)	<0.001
GS, n (%)				
6	N/A	11 (36.7)	N/A	
7	N/A	17 (56.7)	N/A	
8–10	N/A	2 (6.7)	N/A	

s.d.: standard deviation; PSA: prostate specific antigen; p2PSA: [-2]pro prostate specific antigen; PHI: Prostate Health Index; N/A: not available; GS: Gleason score

Table 2: Comparison AUC of various PSA derivatives assuming total PSA as gold standard

Risk factor	AUC (95% CI)	P*
Total PSA	0.4787 (0.3801–0.5774)	-
Free PSA	0.4150 (0.2996–0.5304)	1.000
Free:total PSA ratio	0.4197 (0.2900–0.5493)	1.000
p2PSA	0.6950 (0.5928–0.7973)	0.004
%p2PSA	0.6895 (0.5918–0.7873)	0.046
PHI	0.7937 (0.7113–0.8761)	<0.001

*Bonferroni corrected P value. PSA: prostate specific antigen; p2PSA: [-2]pro prostate specific antigen; PHI: Prostate Health Index; AUC: area under the ROC curve; ROC: receiver operating characteristic; CI: confidence interval

Table 3: Specificity of various PSA derivatives at prespecified sensitivity of 90%

PSA derivatives	Cut-off	Specificity (95% CI)	Number of potentially avoidable biopsies to detect 90% of cancers (percentage of biopsies performed)
Total PSA (ng ml ⁻¹)	≥4.40	17.32 (11.19–25.04)	25 (15.9)
Free PSA	≥0.58	8.66 (4.40–14.97)	14 (8.9)
Free:total PSA ratio	≥0.10	7.09 (3.29–13.03)	12 (7.6)
p2PSA	≥8.6	33.86 (25.70–42.79)	46 (29.3)
%p2PSA	≥1.43	40.94 (32.30–50.02)	56 (35.7)
PHI	≥26.75	58.27 (49.19–66.95)	77 (49.0)
PHI (for GS ≥7 cancer)	≥26.75	55.1 (46.4–63.5)	77 (49.0)

PSA: prostate specific antigen; p2PSA: [-2]pro prostate specific antigen; PHI: Prostate Health Index; CI: confidence interval; GS: Gleason score

The probability of cancer at the higher PHI levels was within the confidence intervals of that reported by the manufacturer. At the lowest risk level (PHI 0–26.9), 80 biopsies or 51% of all the biopsies could have been avoided, with a 5% risk of missing cancer. However, 2 of the 4 cancers detected in this lowest risk group were GS 4 + 3 cancers, one of which was later found to be stage pT3aN0M0 at radical prostatectomy.

As the PHI levels increased, the risk of detecting any cancer and GS ≥7 cancer corresponding increased. At the highest PHI risk group (PHI >55), there was a 42.9% chance of a positive biopsy, all of which were GS ≥7 cancers.

DISCUSSION

There is a need for new biomarkers for early prostate cancer detection

Prostate cancer screening with the PSA test has led to a dramatic reduction in the proportion of men diagnosed with metastatic disease and prostate cancer death rates. Due to the poor specificity of the PSA test, these benefits have come at the cost of a high proportion of unnecessary biopsies and overdiagnosis and overtreatment of indolent cancers.

These problems led the USPSTF (U.S. Preventive Services Task Force) to recommend against prostate cancer screening in all age groups.⁶ This highlights the need for a clinically more useful biomarker for the early detection of prostate cancer.

Validation of the PHI test

The PHI test was approved by the FDA in 2012. The intended use of PHI is to distinguish PCa from benign prostatic conditions in men aged 50 years and older with a total serum PSA between 2 and 10 ng ml⁻¹, and in whom the digital rectal examination is not suspicious for cancer.

A systematic review of studies to date by Abrate *et al.*⁷ and meta-analysis by Wang *et al.*⁸ showed that %p2PSA and PHI were consistently more accurate than standard reference tests in predicting prostate biopsy outcome and could guide prostate biopsy decision making.

Most of the data on %p2PSA and PHI have been based primarily in Caucasian populations, which have a higher incidence of prostate cancer.^{9–12}

Comparison with other Asian studies

Investigators from Hong Kong performed a retrospective evaluation of PHI in Asian men with negative digital rectal examinations, undergoing their initial biopsy with a total PSA of 4–10 ng ml⁻¹.¹³ In comparison to our cohort, which had 19% positive biopsy rate, only 9% of their cohort had a positive biopsy. Nonetheless, similar to our findings, they found that the PHI test had the best performance in predicting the results of the initial biopsy, with an AUC of 0.781 versus 0.547 for total PSA.

Ito *et al.*¹⁴ performed another retrospective evaluation of %p2PSA and PHI in 239 Japanese men with total PSA 2–10 ng ml⁻¹. Abnormal digital rectal examination was not an exclusion criteria in this cohort. They found that at sensitivity levels of 70%–95%, PHI showed superior specificity in predicting prostate cancer compared to total PSA and %fPSA.

Na *et al.*¹⁵ prospectively evaluated p2pSA and PHI in a large hospital-based cohort of 636 Chinese men. However, almost 30% of

Table 4: Performance of PHI test according to manufacturer banding of PHI levels

PHI level	Total (n=157)	GS ≥ 7 cancer (n=19) (%)	All cancer (n=30) (%)	No cancer (n=127) (%)	Probability of cancer - from manufacturer brochure (%)	95% CI
0–26.9	80	2 (2.5)	4 (5)	76 (95)	9.8	5.2–15.4
27.0–35.9	35	2 (5.7)	10 (28.6)	25 (71.4)	16.8	11.3–22.2
36.0–54.9	35	12 (34.2)	13 (37.1)	22 (62.9)	33.3	26.8–39.9
≥ 55.0	7	3 (42.9)	3 (42.9)	4 (57.1)	50.1	39.8–61.0

PHI: Prostate Health Index; GS: Gleason score; CI: confidence interval

Table 5: Avoidable biopsies at different PHI thresholds and probability of missed prostate cancers, or GS ≥ 7 cancers

PHI level	Number of avoidable biopsies at this threshold (%)	Number of GS ≥ 7 cancer missed (n=19) (%)	Number of any cancer missed (n=30) (%)
≥ 27.0	80 (51.0)	2 (2.5)	4 (5.0)
≥ 36.0	115 (73.2)	4 (3.5)	14 (12.2)
≥ 55.0	150 (95.5)	16 (10.7)	27 (18.0)

PHI: Prostate Health Index; GS: Gleason score

this cohort had positive DRE, and the total PSA ranged from 0.04 to 2006 ng ml⁻¹. Not surprisingly, the positive biopsy rate was as high as 43.1%.

When they divided their cohort to those with total PSA 2–10 ng ml⁻¹, 10.1–20 ng ml⁻¹ and more than 20 ng ml⁻¹, the AUC of total PSA versus PHI was 0.53, 0.58, 0.80 versus 0.73, 0.81, 0.90, respectively. Thus, while PHI was superior to total PSA at all total PSA levels, PHI showed the biggest advantage over PSA in the gray zone below 10 ng ml⁻¹.

Our results showed that PHI was the best performer in predicting prostate cancer in the initial biopsy. At 90% sensitivity, the specificity of PHI at 58.27% was 3 times better than that of total PSA at 17.32%. Together with the retrospective data provided by the Japanese and Hong Kong cohorts, our work validates the use of %p2PSA and PHI in Asian men with total PSA in the diagnostic “gray zone” of 4–10 ng ml⁻¹.

Avoiding unnecessary biopsies

In our cohort, a biopsy threshold at PHI ≥ 27.0 , would have avoided 80 or 51% of biopsies in this cohort, at a 2.5% risk of missing a potentially aggressive cancer (GS ≥ 7 or more) (Table 5).

This should be taken in the perspective that the prostate cancer detection rate below the usual total PSA biopsy threshold of 4 ng ml⁻¹ can be as high as 20%, with up to 24% having GS ≥ 7 cancer.¹⁶

This was also seen in an observational, prospective, multicenter European cohort¹⁷ (PROMetheus Project). This study involved 646 patients from five European urology centers with total PSA of 2 to 10 ng ml⁻¹, who were subjected to initial prostate biopsy for suspected PCa. They found p2PSA, %p2PSA, and PHI significantly increased the accuracy of the base multivariable model by 6.4%, 5.6%, and 6.4%, respectively ($P < 0.001$). At 90% sensitivity, the PHI cut-off of 27.6 could result in the avoidance of 100 biopsies (15.5%), with 26 cancers (9.8%) being overlooked (23 with GS 6, 3 with GS 3 + 4).

Loeb *et al.*¹⁸ similarly showed that in a cohort of U.S men with total PSA 4–10 ng ml⁻¹, a PHI cut-off of 28.6, could avoid approximately 30% of biopsies, while missing 10% of GS ≥ 7 PCa.

Association with clinically significant prostate cancer

We looked at whether PHI could reliably detect clinically significant prostate cancer (GS ≥ 7) (Table 4). We found that as PHI levels increased, the detection of GS ≥ 7 cancers corresponding increased, with PHI level 36.0 and more detecting the majority of GS ≥ 7 cancers in the cohort. This has also been recently shown by de la Calle *et al.*¹⁹ in a large multicenter European cohort.

Furthermore, there are studies that have shown that PHI selectively identifies clinically significant PCa in biopsies,^{12,18} and can help predict for more aggressive pathological features at radical prostatectomies.^{20,21}

Cost-effectiveness of PHI in the early detection of prostate cancer

It would be expected that the three-fold greater specificity of PHI compared to PSA, with the resulting decrease in the cost of unnecessary biopsies, can lead to healthcare cost savings.

In two studies, Nichol *et al.* evaluated the cost-effectiveness of PHI. In the first study,²² the authors constructed two budget impact models using PSA cut-off values of ≥ 2 ng ml⁻¹ (model #1) and ≥ 4 ng ml⁻¹ (model #2) for recommending a prostate biopsy in a hypothetical health plan with 100 000 male members aged 50–75 years old. The budgetary impact on the 1-year expected total costs for PCa detection was calculated. Adding PHI to the current PSA screening strategies increased the total costs of blood tests by \$51 524 (model #1) and \$13 611 (model #2). However the reduction of both true- and false-positive, which may reduce the number of office visits, laboratory tests and prostate biopsies, led to higher cost savings in model #1 (\$356 647) than in model #2 (\$94 219), with 92% of the savings realized by the reduction of unnecessary biopsies.

In the second study,²³ the same group evaluated the cost-effectiveness of early PCa detection with PHI associated with a PSA test compared with the PSA test alone from a United States of America societal perspective. Over 25 annual screening cycles, the strategy of PSA plus PHI was estimated to save \$1199 or \$443 with an expected gain of 0.08 or 0.03 quality-adjusted life years per person for PSA thresholds of ≥ 2 and ≥ 4 ng ml⁻¹, respectively. Because the strategy of PSA plus PHI was expected to increase the number of true-positive tests while reducing false-positives in men aged 50–75 years, the authors suggested that the increased total costs of adding PHI could be offset by reducing unnecessary prostate biopsies.

At first glance, this may not appear to be large savings, but if the cost of avoiding the attendant morbidities from prostatic biopsies, especially that of urosepsis, it can be expected that these savings will be increased.

PHI in clinical guidelines

The use of the PHI has been included in the National Comprehensive Cancer Network (NCCN, Beckman Coulter) guidelines on early detection of prostate cancer version 2.2015. Consistent with the current literature, they have recommended that biomarkers that improve the specificity of detection should not be used as first-line screening tests.

However, for patients who meet either PSA or DRE standards for consideration of biopsy, but for whom the patient and/or the physician wish to further define the probability of high-grade cancer, the guidelines have stated that PHI > 35 (which provides an estimate of the probability of high-grade prostate cancer) is potentially informative in patients who have never undergone biopsy or after a negative biopsy.

The 2015 European Association of Urology (EAU) guideline for prostate cancer has stated that the PHI test may have a role in monitoring men under active surveillance. However, the guideline has not made a recommendation for its use in men not diagnosed with prostate cancer.

Our study also shows that PHI ≥ 36.0 was associated with a marked increase chance of detecting GS ≥ 7 prostate cancer compared to PHI < 36.0 , detecting 15/19 (79%) of the GS ≥ 7 cancers (Table 5), supporting the above recommendation by the NCCN.

Strengths and limitations of study

The strengths of our study include the prospectively enrolled source population from two centers. All blood samples were optimally managed according to the Semjonow *et al.*²⁴ guidelines. All men had a histological diagnosis for endpoint assessment and in a direct comparison PHI was shown to outperform its individual components.

The limitations of our study are as follows. First, the relatively small sample size of the study may have limited the statistical significance of our findings. Second, we followed patients only through the index biopsy, and it is likely that some of the patients had false-negative prostate biopsies, given the known sampling error of a needle biopsy for the detection of cancer and the underdetection of high-grade prostate cancer. Finally, no correlation with pathologic outcome in patients who underwent RP after diagnosis was reported.

While we have shown that %p2PSA and PHI perform well as a reflex test in men in the PSA diagnostic gray zone, the potential role of %p2PSA and PHI as a screening test to replace PSA screening was not examined, as these findings do not extend to men who have not been prescreened by PSA. This is a flaw shared by all the other studies in the literature validating the use of %P2PSA and PHI.

To conclusively prove that %p2PSA and PHI are superior screening tools, prospective studies based on direct PCa screening by %p2PSA and PHI are needed.

CONCLUSIONS

Similar to studies in mainly Caucasian populations, we have prospectively shown that %p2PSA and PHI greatly outperform total and free to total PSA ratio, in the detection of prostate cancer at first biopsy in an Asian population with serum PSA between 4 and 10 ng ml⁻¹.

PHI is useful in reflex testing in men in the diagnostic gray zone of serum PSA between 4 and 10 ng ml⁻¹, aiding decision making about prostate biopsies, and can also avoid a significant proportion of unnecessary biopsies. Its potential to replace total PSA as a cost-effective prostate cancer screening tool needs to be validated in prospective trials in cohorts not selected by PSA.

AUTHORS CONTRIBUTIONS

LGT conceived and designed the study, drafted and revised the manuscript. YKT participated in the design of the study, data acquisition and critical review of the manuscript. BCT conducted statistical design of the study and data analysis. KMT, RCH and FSH participated in data acquisition. VG conceived and participated in the design of the study. TT performed the review of the prostate biopsies. HYT participated in data acquisition and critical review of the manuscript. EC participated in designing the study and critical review of the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS

None of the authors declare competing financial interests.

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