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Enzymes and Protein Markers

The Combination of Human Glandular Kallikrein and Free Prostate-specific Antigen (PSA) Enhances Discrimination Between Prostate Cancer and Benign Prostatic Hyperplasia in Patients with Moderately Increased Total PSA

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Background: **Prostate-specific antigen (PSA) is the most reliable tumor marker available and is widely used for the diagnosis and management of prostate cancer. Unfortunately, PSA cannot distinguish efficiently between benign and malignant disease of the prostate, especially within the range of 4–10 μg/L. Among the refinements developed to enhance PSA specificity is the free/total PSA ratio, which is useful in discriminating between the two diseases within the diagnostic "gray zone". Recent data indicate that human glandular kallikrein (hK2), a protein with high homology to PSA, may be an additional serum marker for the diagnosis and monitoring of prostate cancer.**

Methods: **We analyzed 206 serum samples (all before treatment was initiated) from men with histologically confirmed benign prostatic hyperplasia (n = 100) or prostatic carcinoma (n = 106) with total PSA in the range of 2.5–10 μg/L. Total and free PSA and hK2 were measured with noncompetitive immunological procedures. Statistical analysis was performed to investigate the potential utility of the various markers or their combinations in discriminating between benign prostatic hyperplasia and prostatic carcinoma.**

Results: **hK2 concentrations were not statistically different between the two groups of patients. There was a strong positive correlation between hK2 and free PSA in the whole patient population. hK2/free PSA ratio (area under the curve = 0.69) was stronger predictor of prostate cancer than the free/total PSA ratio (area under the curve = 0.64). At 95% specificity, the hK2/free PSA ratio identified 30% of patients with total PSA between 2.5–10 μg/L who had cancer. At 95% specificity, the hK2/free PSA ratio identified 25% of patients with total PSA between 2.5 and 4.5 μg/L who had cancer.**

Conclusions: **Our data suggest that hK2 in combination with free and total PSA can enhance the biochemical detection of prostate cancer in patients with moderately increased total PSA concentrations. More specifically, the hK2/free PSA ratio appears to be valuable in identifying a subset of patients with total PSA between 2.5 and 4.5 μg/L who have high probability of cancer and who should be considered for biopsy.**

In the past decade, prostate cancer (CaP)[3] has emerged as one of the most common diseases among men over 50 years of age, particularly in Western societies. CaP is the most frequently diagnosed cancer in men in the United States, and its mortality rate is second only to lung cancer. Therefore, early diagnosis and monitoring of CaP is an important priority. This clinical need is considerably

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[3] Nonstandard abbreviations: CaP, prostate cancer; PSA, prostate-specific antigen; BPH, benign prostatic hyperplasia; hK2, human glandular kallikrein; AUC, area under the curve; CI, confidence interval; and DRE, digital rectal examination.

enhanced by the measurement of serum prostate-specific antigen (PSA), the most reliable tumor marker established to date *(1)*. However, the analysis of total PSA in serum lacks sensitivity and specificity to provide optimal detection of early CaP, especially in the 4–10 μg/L "gray zone", where it cannot discriminate between patients with CaP and benign prostatic hyperplasia (BPH). In addition, concentrations <4 μg/L do not always indicate absence of CaP because 20–30% of men with organ-confined but clinically significant CaP have serum PSA concentrations within the reference interval *(2)*.

To improve the clinical value of PSA, different methods have been developed, including PSA density *(3, 4)*, PSA velocity *(5)*, and age-specific reference ranges *(6)*. Furthermore, it has been shown that measurement of the different molecular forms of serum PSA *(7, 8)* helps to discriminate between CaP and BPH. PSA is present in serum predominantly bound to proteinase inhibitors (α_1-antichymotrypsin and α_2-macroglobulin), but there is also a fraction of free PSA, an uncomplexed form that is enzymatically inactive. Recent studies indicate that the free/total PSA ratio in serum is lower in patients with CaP than in patients with BPH, thus enhancing the clinical usefulness of PSA testing in CaP screening programs [Scorilas et al., submitted for publication; and Refs. *(9–13)*].

Human glandular kallikrein (hK2) belongs to the same family of serine proteases as PSA (human kallikrein gene family) and displays a strong structural homology to PSA. Moreover, the two kallikreins share many biochemical properties: they are both primarily localized in the prostate and they are androgen regulated *(14)*. Findings that hK2 cleaves proPSA (244 residues) to generate enzymatically active PSA (237 residues) suggests a physiological role of hK2 in the regulation of PSA *(15)*. These striking similarities led to the investigation of the potential role of hK2 as an additional prostate tumor marker, which may have the potential to enhance the accuracy of diagnosis and prognosis of current PSA testing.

In this study, we measured serum total and free PSA and hK2 in a group of patients with BPH or CaP, in the intermediate range of total PSA of 2.5–10 μg/L. We further analyzed the value of these variables, as well as their various combinations in discriminating between these two clinical entities.

Materials and Methods

PATIENTS

Included in this study were serum samples from 206 male patients, 100 with BPH (median age, 65 years) and 106 with CaP (median age, 67 years), histologically confirmed by biopsy in all cases. All patients had total PSA concentrations between 2.5 and 10 μg/L. They were patients who participated in a CaP screening program, and they were biopsied because of slightly to moderately increased serum PSA. All sera were stored at −70 °C until analysis.

IMMUNOASSAYS

Concentrations of PSA were measured by the Tandem total PSA and free PSA monoclonal antibody-based assays (Hybritech) *(16)*. A new, time-resolved immunofluorometric assay, recently developed in our laboratory, was used to measure serum hK2 concentrations *(17)*. Briefly, the hK2 assay uses a mouse monoclonal anti-hK2 capture antibody [coded G586, supplied by Hybritech (San Diego, CA) and raised against recombinant hK2], a biotinylated mouse monoclonal detection antibody (coded 8311; Diagnostic Systems Laboratories) and alkaline phosphatase-labeled streptavidin. We measured the alkaline phosphatase activity by adding the substrate difluni-sal phosphate, incubating for 10 min, and then adding a Tb^{3+}-EDTA developing solution. The fluorescence was measured on a Cyberfluor 615 Immunoanalyzer (MDS Nordion). The hK2 assay has a detection limit of 0.006 μg/L and has <0.2% cross-reactivity to PSA. A full description of the method and its evaluation has been published elsewhere *(17)*.

STATISTICAL ANALYSIS

Several functions between PSA and hK2 were calculated, and descriptive statistics for these variables were performed for each group of patients. Because the distributions of total PSA, free PSA, and hK2 in the BPH and CaP patients were not gaussian, the analyses of differences between these variables in the two groups were performed with the nonparametric Mann–Whitney *U*-test. Relationships between different variables were assessed by the Spearman correlation coefficient. ROC curves were constructed for total PSA and the free/total PSA and hK2/free PSA ratios, plotting sensitivity vs (1 − specificity), and the areas under the ROC curves (AUCs) were

Table 1. Descriptive statistics of various variables in serum of 100 BPH patients.

			Percentile				
Variable	Mean ± SE	Range	5	25	50	75	95
Total PSA, μg/L	5.23 ± 0.16	2.61–9.55	3.02	4.25	4.91	6.05	8.47
Free PSA, μg/L	1.03 ± 0.056	0.31–4.93	0.43	0.68	0.91	1.21	1.96
Free/total PSA	0.20 ± 0.008	0.062–0.53	0.088	0.13	0.19	0.24	0.32
hK2, μg/L	0.73 ± 0.043	0.032–1.80	0.099	0.39	0.68	0.97	1.56
hK2/free PSA	0.77 ± 0.041	0.030–1.72	0.19	0.43	0.75	1.05	1.56

Table 2. Descriptive statistics of various variables in serum of 106 CaP patients.

Variable	Mean ± SE	Range	Percentile 5	25	50	75	95
Total PSA, μg/L	4.99 ± 0.17	1.78–10.0	2.67	4.15	4.61	5.58	8.53
Free PSA, μg/L	0.77 ± 0.043	0.15–2.59	0.26	0.43	0.69	0.97	1.84
Free/total PSA	0.16 ± 0.007	0.020–0.41	0.061	0.098	0.15	0.21	0.27
hK2, μg/L	0.79 ± 0.048	0.031–2.79	0.097	0.44	0.72	1.04	1.67
hK2/free PSA	1.18 ± 0.075	0.051–5.02	0.20	0.61	1.02	1.59	2.59

calculated. Univariate and multivariate unconditional logistic regression models were developed to evaluate the ability of PSA and hK2 concentrations to predict the presence of CaP. For all analyses, $P < 0.05$ was considered statistically significant.

Results

Total PSA, free PSA, and hK2 were measured in 206 serum samples from 106 patients with CaP and 100 patients with BPH. The descriptive statistics for these variables as well as for the hK2/total PSA, hK2/free PSA, and hK2/(free/total PSA) ratios are presented in Tables 1 and 2.

Total PSA values were 2.61–9.55 μg/L in BPH patients, with a mean ± SE of 5.23 ± 0.16 μg/L, and 1.78–10 μg/L in CaP patients, with a mean ± SE of 4.99 ± 0.17 μg/L. No statistically significant difference was found in total PSA concentrations between the two groups of patients. Free PSA concentrations were 0.31–4.93 μg/L (mean ± SE, 1.03 ± 0.056 μg/L) and 0.15–2.59 μg/L (mean ± SE, 0.78 ± 0.043 μg/L) in patients with benign and malignant prostatic disease, respectively ($P < 0.001$). The mean ± SE of the free/total PSA ratio was 0.20 ± 0.008 (range, 0.062–0.53) in patients with BPH and 0.16 ± 0.007 (range, 0.02–0.41) in patients with CaP ($P < 0.001$). hK2 was detected in all samples from BPH patients and ranged from 0.032 to 1.80 μg/L, the mean ± SE being 0.73 ± 0.043 μg/L. In the CaP patients, hK2 concentrations were between 0.031 and 2.79 μg/L, with a mean ± SE value of 0.79 ± 0.048 μg/L. The distribution of hK2 values was not different between the two groups of patients. In general, the concentration of free PSA was lower, whereas the hK2 concentration was slightly increased, in the CaP patients when compared with the respective values in the BPH patients (Fig. 1). Several functions between PSA and hK2 (ratios, logarithmic ratios, differences) were calculated and evaluated for their discriminatory potential between BPH and CaP (data not shown). We observed that the hK2/free PSA ratio had good discriminatory power, and we further pursued its analysis. The mean ± SE value of the hK2/free PSA ratio was 0.77 ± 0.041 in patients with BPH, whereas it was 1.18 ± 0.075 in patients with CaP ($P < 0.001$).

In the group of patients with BPH, we found a strong positive correlation between hK2 and free PSA concentrations (Spearman correlation coefficient $r_s = 0.512$; $P < 0.001$), as well as between hK2 and free/total PSA ratio ($r_s = 0.413$; $P < 0.001$). hK2 and total PSA concentrations also correlated but less strongly ($r_s = 0.215$; $P = 0.031$). In

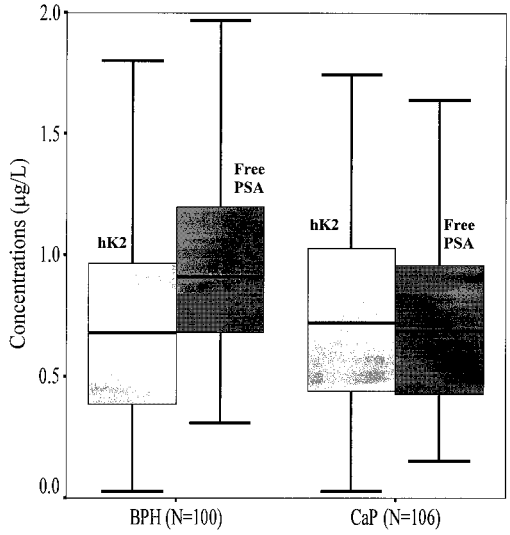

Fig. 1. Distribution of hK2 and free PSA in BPH and CaP patients. The *box-plots* display the quartiles.

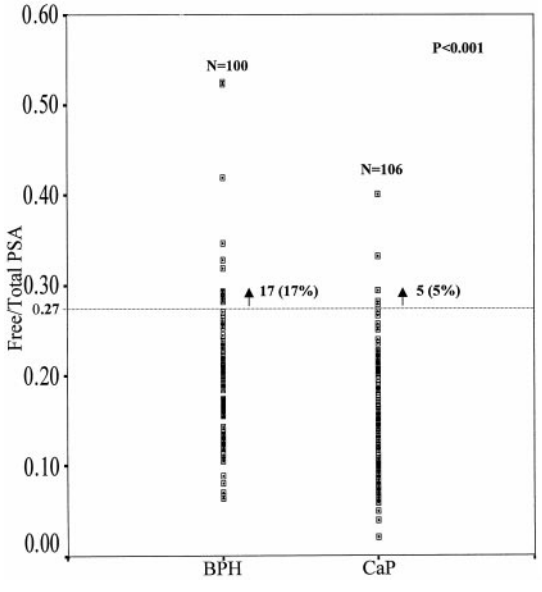

Fig. 2. Distribution of free/total PSA ratios in BPH and CaP patients. At 95% sensitivity and 17% specificity, the free/total PSA ratio is 0.27. *P* was determined by the Mann–Whitney *U*-test.

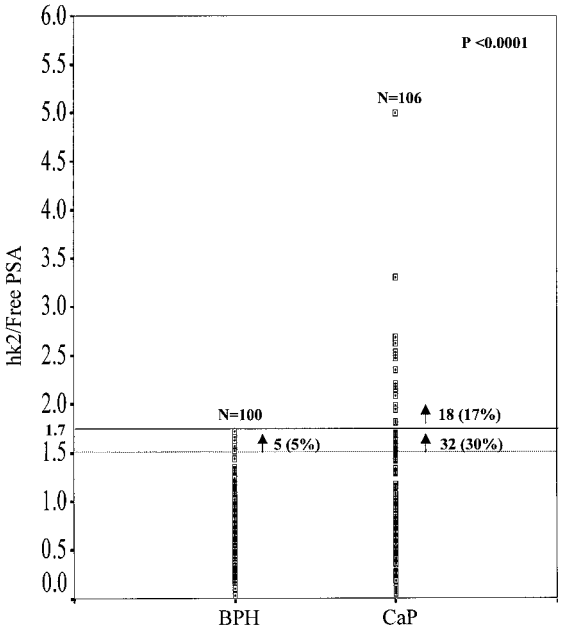

Fig. 3. Distribution of hK2/free PSA ratios in BPH and CaP patients.

At 30% sensitivity and 95% specificity, the hK2/free PSA ratio is 1.5. *P* was determined by the Mann–Whitney *U*-test.

patients with CaP, a positive correlation between hK2 and free PSA (r_s = 0.429; P <0.001), as well as between hK2 and the free/total PSA ratio (r_s = 0.372; P <0.001) was also found. On the other hand, there was no significant correlation between hK2 and total PSA. The hK2/free PSA ratio did not correlate with the free/total PSA ratio in the BPH patients, whereas the two ratios showed a negative correlation in the CaP patients (r_s = −0.26; P = 0.007).

The distributions of the free/total PSA ratios in the two groups of patients are presented in Fig. 2. With a cutoff value of 0.27 (95% sensitivity, 17% specificity), 17% of biopsies could have been avoided in BPH patients, whereas 5% of the cancers would have been missed. In Fig. 3, the distribution of hK2/free PSA ratios in the same

Table 3. Univariate analysis of BPH and CaP patients for predicting the presence of CaP, using unconditional logistic regression modeling.

Covariate	Crude risk ratio	95% CI	*P*
Total PSA[a]	0.92	0.77–1.08	0.31
Free PSA[a]	0.32	0.16–0.63	0.0011
Free/total PSA ratio[a]	0.30	0.12–0.75	0.0098
hK2[a]	1.31	0.72–2.37	0.37
hK2/free-PSA ratio[a]	3.44	1.95–6.05	0.001
DRE			
Normal	1.00		
Abnormal	2.11	0.96–4.65	0.063
Age[a]	1.03	0.99–1.08	0.081

[a] Test for trend.

Table 4. Multivariate analysis of BPH and CaP patients for predicting the presence of CaP, using unconditional logistic regression modeling.

Covariate	Crude risk ratio	95% CI	*P*
Total PSA[a]	1.14	0.91–1.43	0.24
Free/total PSA ratio[a]	0.41	0.19–0.86	0.019
hK2/free-PSA ratio[a]	2.93	1.51–5.71	0.0015
DRE			
Normal	1.00		
Abnormal	1.98	0.88–4.41	0.096
Age[a]	1.05	0.99–1.11	0.061

[a] Test for trend.

population of patients is shown. At a putative cutoff of 1.5 (95% specificity), the sensitivity was 30%.

ROC analyses were performed to show the relative potential of the total PSA concentration and the free/total PSA and hK2/free PSA ratios in the discrimination of CaP and BPH. The free/total PSA ratio [AUC, 0.64; 95% confidence intervals (CI), 0.57–0.72] was significantly more predictive of cancer than total PSA concentration alone (AUC, 0.56; 95% CI, 0.48–0.64), as reported previously *(7–13)*. Interestingly, the hK2/free PSA ratio is more discriminative between the two clinical conditions (AUC, 0.69; 95% CI, 0.61–0.76). ROC analysis for the hK2/(free/total PSA) ratio showed that this variable also has differentiating power (AUC, 0.67; 95% CI, 0.60–0.74).

Univariate logistic regression models were developed to evaluate the value of PSA and hK2 concentrations in the discrimination between BPH and CaP patients (Table 3). These models demonstrated that patients with high hK2/free PSA ratios were at increased risk to have CaP (crude odds ratio, 3.44; 95% CI, 1.95–6.05). In the multivariate analysis, the logistic regression models were adjusted for total PSA, the free/total PSA and hK2/free PSA

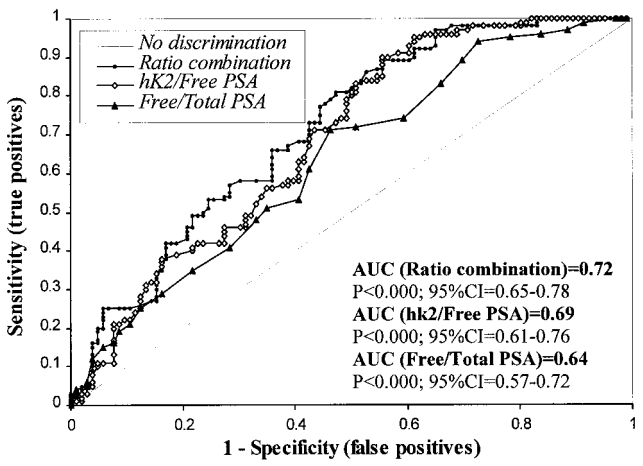

Fig. 4. ROC curves for the free/total PSA ratio, the hK2/free PSA ratio, and the combination of the two ratios.

The ROC curves demonstrate the relative potential of each variable in the discrimination of BPH from CaP in the total PSA range of 2.5–10 μg/L.

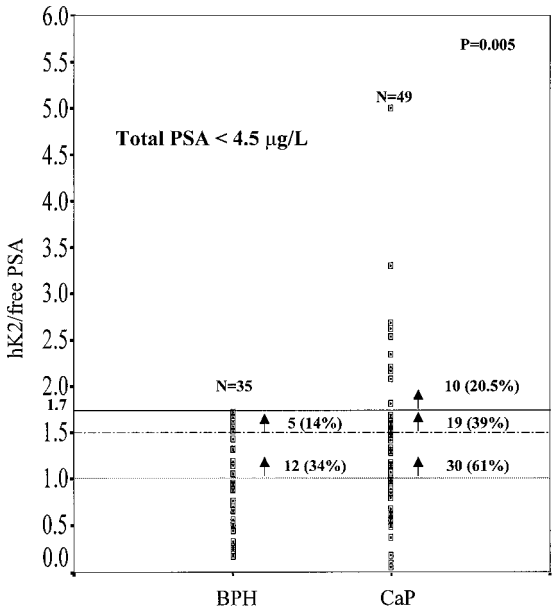

Fig. 5. Distribution of hK2/free PSA ratios in BPH and CaP patients with total PSA <4.5 μg/L.

At 39% sensitivity and 86% specificity, the hK2/free PSA ratio is 1.5, whereas at 61% sensitivity and 34% specificity, the ratio is 1.0. *P* was determined by the Mann–Whitney *U*-test.

ratios, digital rectal examination (DRE), and patient age; all of these covariates were used as continuous variables, except the DRE, which was used as a categorical variable. The hK2/free PSA ratio was an independent factor for discriminating between BPH and CaP patients (crude odds ratio, 2.93; 95% CI, 1.51–5.71). The free/total PSA ratio added significantly to the prognostic power of this multivariate model (crude odds ratio, 0.41; 95% CI, 0.19–0.86; Table 4).

To further investigate the discriminatory value of the hK2/free PSA ratio in relation to the free/total PSA ratio, another logistic regression model was developed that adjusted for only these two ratios. This model also confirmed the higher potential of the hK2/free PSA ratio in discriminating BPH from CaP patients (crude odds ratio for hK2/free PSA, 3.35; 95% CI, 1.89–5.94; crude odds ratio for free/total PSA, 0.47; 95% CI, 0.23–0.96). We calculated log likelihood scores for this multivariate logistic regression model, which incorporates both of the ratios, for each patient. From these data, by picking different thresholds for the regression function values, we devised a ROC curve (Fig. 4) that shows the added value of using both ratios together in a multivariate function (AUC, 0.72; 95% CI, 0.65–0.78).

In Fig. 5, the distributions of hK2/free PSA ratios in patients with BPH or CaP and total PSA <4.5 μg/L are presented. At a specificity of 95%, the sensitivity of the ratio in ruling-in cancer was 25%. Among 42 patients who had total PSA <4.5 μg/L and a hK2/free PSA ratio of ≥1.0, 30 had cancer and 12 had BPH. However, among 42 patients who have total PSA <4.5 μg/L and a hK2/free PSA ratio <1.0, 19 had CaP and 23 had BPH.

Discussion

The ratio of free to total PSA (percentage of free PSA) in serum has been reported to be significantly higher in individuals with BPH than in CaP patients *(7)*, even at PSA concentrations <10 μg/L, where the measurement of total PSA fails to discriminate efficiently between the two clinical conditions. Large clinical studies established that the percentage of free PSA could be utilized to reduce by ~20% the number of unnecessary biopsies while maintaining a high clinical sensitivity for the detection of CaP *(9–13)*.

In the current study, we analyzed serum samples from 206 men, 50–75 years of age, with total PSA values of 2.5–10 μg/L. Recently, Catalona et al. *(13)* reported that a 25% free PSA cutoff detected 95% of cancers while avoiding 20% of biopsies in a cohort of patients with total PSA concentrations of 4.0–10.0 μg/L. This is in agreement with the data presented here, where at the same sensitivity (95%), the specificity was 17%, with the free/total PSA ratio being 27% (Fig. 2). The AUC was significantly higher for the free/total PSA ratio (0.64) than for total PSA (0.56), confirming the data published previously *(11, 13)*.

Early studies demonstrated that hK2 is present in serum of patients with increased PSA, suggesting that it may be a new CaP marker *(18, 19)*. Darson et al. *(20)* found that hK2 expression is tumor associated, in contrast to PSA, which is expressed at higher concentrations in noncancerous prostate cells. Studies are now underway to investigate the molecular form(s) of hK2 that will provide the most useful clinical information with respect to both screening and staging of CaP. Recent data show that the concentration of the precursor form of hK2 (prohK2) is increased in prostatic cancer tissue compared with BPH or healthy tissue *(21)*, as well as in the serum of patients with prostatic disease *(22)*.

In our study, we detected substantial amounts of hK2 in all serum samples. The hK2 absolute concentrations that we report are approximately ninefold higher than values reported previously by others *(23)*. We speculate, as also mentioned elsewhere *(24)*, that these differences may be attributable to standardization and/or to different recognition of hK2 molecular forms by the assays used. We found no statistically significant difference in hK2 values between BPH and CaP patients. A positive correlation between hK2 and free PSA was observed in the whole population of patients. hK2 also correlated with total PSA in patients with BPH, whereas this was not the case in patients with CaP. Our data suggest that hK2 alone cannot discriminate between benign and malignant prostatic diseases in patients with PSA values within the diagnostic gray zone. However, other groups have reported that hK2 concentrations differ significantly between the two groups of patients and that they are not directly proportional to PSA serum concentrations in

either BPH or cancer *(25)*. The fact that we have analyzed a selected population of patients (with moderately increased PSA) may account, to some extent, for these differences.

Becker et al. *(26)* recently reported that the hK2/free PSA ratio is higher in CaP patients than non-cancer subjects. To examine the potential clinical utility of this ratio in the discrimination between BPH and CaP in patients with moderately increased PSA, we performed statistical analysis of this variable. The results showed that the hK2/free PSA ratio is significantly higher in a proportion of CaP patients, suggesting that this ratio may be a potential new prostatic marker. Of interest is the finding that all BPH patients had hK2/free PSA ratios below 1.7 (Fig. 3). We also constructed ROC curves (Fig. 4) and performed multivariate analysis to further investigate the potential of the hK2/free PSA ratio in the differentiation between the two diseases (Table 4). This ratio appears to have a greater potential in discriminating the CaP patients than the free/total PSA ratio. Furthermore, an additional multivariate logistic regression model was developed to assess the diagnostic value of these two ratios combined. The ROC curve derived (Fig. 4) demonstrates the superiority of using the combination of the two ratios. This variable seems promising and may have real clinical merit in the future, after larger clinical studies establish cutoff values for both ratios.

Among the other functions of hK2 and PSA that we analyzed, the hK2/(free/total PSA) ratio showed a discriminatory efficacy slightly inferior to that of the hK2/free PSA ratio. However, we observed that all patients with hK2/free PSA ratios >1.5 and hK2/(free/total PSA) ratios>7.3 had CaP (data not shown). The combination of these two ratios was able to discriminate 37% of CaP patients with 95% specificity. Therefore, it seems that this concept has the potential to function as a new, valuable marker in the discrimination of CaP patients with moderately increased total PSA ranges.

It is now recommended that all patients with total PSA >10 μg/L and all patients with total PSA >4 μg/L and a percentage of free PSA <25% be biopsied *(13)*. Thus, even if the hK2/free PSA ratio is >1.7 in this group of patients, suggesting that they are very likely to have cancer (Fig. 3), they will still be biopsied for confirmation. Consequently, the number of unnecessary biopsies will not be reduced if the hK2/free PSA ratio is used in patients with total PSA >4 μg/L. However, when total PSA is <4 μg/L, biopsy is not recommended *(13)*. In Fig. 5 we present data supporting the notion that patients with an increased hK2/free PSA ratio and total PSA <4.5 μg/L are at a substantially increased risk of having CaP. Although our data are preliminary and need confirmation, it seems that these patients with total PSA between 2.5 and 4.5 μg/L who have hK2/free PSA ratios >1.0 may be good candidates for biopsy because they have a greater than 35% chance of having cancer (30 patients out of a total of 84 patients). Of 42 biopsies performed in this group of patients with total PSA between 2.5 and 4.5 μg/L and hK2/free PSA ratios >1, 30 (71%) will be diagnosed as cancer. We here draw attention to the fact that our groups of patients have been selected and do not represent the distribution of all men in a general population who have total PSA between 2.5 and 4.5 μg/L. In our population, 49 of 84 (58%) have cancer, whereas it is known that the prevalence of cancer in men with total PSA between 2.5 and 4.5 μg/L is ~20% *(13)*.

Kwiatkowski et al. *(27)* also reported that the hK2/free PSA ratio has a potential discriminatory power in prostatism patients within the diagnostic gray zone of total PSA 4–10 μg/L. In that report, hK2 was determined by an indirect assay, which had a functional sensitivity limit almost 10 times lower than ours. This explains the variations in hK2 concentrations and in hK2/free PSA ratios reported in the two studies. Another difference was the small number of subjects used for the generation of their ROC curves, leading to a possible overestimation of the discriminating power of hK2/free PSA ratio.

In conclusion, we investigated the potential utility of hK2 in the discrimination between CaP and BPH in the total PSA range of 2.5–10 μg/L. We found that in this selected population of patients, hK2 could play a useful diagnostic role, but only in combination with free PSA and free/total PSA ratio. This approach could identify a subgroup of patients with low total PSA (e.g., between 2.5 and 4.5 μg/L) who probably would not be biopsied on the basis of their total PSA alone but who are at high risk of having cancer and should be considered for biopsy. This new diagnostic modality is complementary to the free/total PSA ratio because the former has utility in ruling-in CaP, whereas the latter has utility in ruling-out CaP. Larger multicenter clinical trials, including a greater number of controls, are necessary to further evaluate the use of this new approach in the differential diagnosis of CaP and BPH and to establish putative cutoff values.

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