Published in final edited form as:

Int J Cancer. 2009 February 1; 124(3): 659–663. doi:10.1002/ijc.23983.

Evaluation of molecular forms of PSA and human kallikrein 2 in predicting biochemical failure following radical prostatectomy

Sven Wenske^{1,2,#}, Ruslan Korets^{1,#}, Angel M. Cronin³, Andrew J. Vickers³, Martin Fleisher⁴, Howard I. Scher⁵, Kim Pettersson⁶, Bertrand Guillonneau¹, Peter T. Scardino¹, James A. Eastham¹, and Hans Lilja^{1,4,5}

¹Department of Surgery (Urology Service), Memorial Sloan-Kettering Cancer Center, New York, NY

²Department of Urology, Marienhospital Herne, Ruhr-University, Bochum, Germany ³Department of Epidemiology and Biostatistics, Memorial Sloan-Kettering Cancer Center, New York, NY

⁴Department of Clinical Laboratories, Memorial Sloan-Kettering Cancer Center, New York, NY

⁵Department of Medicine (Genitourinary Oncology Service), Memorial Sloan-Kettering Cancer Center, New York, NY ⁶Department of Biotechnology, University of Turku, Turku, Finland

Abstract

Most pretreatment risk-assessment models to predict biochemical recurrence (BCR) after radical prostatectomy (RP) for prostate cancer rely on total prostate-specific antigen (PSA), clinical stage, and biopsy Gleason grade. We investigated whether free PSA (fPSA) and human glandular kallikrein-2 (hK2) would enhance the predictive accuracy of this standard model. Preoperative serum samples and complete clinical data were available for 1,382 patients who underwent RP for localized prostate cancer from 1993 – 2005. A case-control design was utilized, and conditional logistic regression models were used to evaluate the association between preoperative predictors and BCR after RP. We constructed multivariable models with fPSA and hK2 as additional preoperative predictors to the base model. Predictive accuracy was assessed with the area under the ROC curve (AUC). There were 146 BCR cases; the median follow up for patients without BCR was 3.2 years. Overall, 436 controls were matched to 146 BCR cases. The AUC of the base model was 0.786 in the entire cohort; adding free PSA and hK2 to this model enhanced the AUC to 0.798 ($p=0.053$), an effect largely driven by free PSA. In the subgroup of men with tPSA ≤ 10 ng/ml (48% of cases), adding free PSA and hK2 enhanced the AUC of the base model to a similar degree (from 0.720 to 0.726, $p=0.2$). Free PSA is routinely measured during prostate cancer detection. We suggest that the role of fPSA in aiding preoperative prediction should be investigated in further cohorts.

Keywords

free PSA; PSA; human kallikrein 2; prostate cancer; biochemical recurrence; radical prostatectomy

*Correspondence to: Hans Lilja, MD, PhD Department of Surgery (Urology) Memorial Sloan-Kettering Cancer Center 1275 York Avenue, Box 213 New York, NY 10021 Phone: +1-646-422-6982 FAX: +1-646-422-2379 liljah@mskcc.org.

#The first 2 authors contributed equally to this work.

Financial disclosures: Dr. Hans Lilja holds patents for free PSA and hK2 assays.

Summary Statement

Our study suggests that the predictive accuracy of a standard preoperative risk-prediction model for biochemical recurrence after radical prostatectomy for localized prostate cancer may be enhanced with the addition of free PSA and human kallikrein 2. The role of free PSA – which is routinely measured during detection of prostate cancer – should be investigated in further cohorts.

INTRODUCTION

Other than its established clinical application for the early detection of prostate cancer, prostate-specific antigen (PSA) represents one of the key variables in currently available prognostic models for clinically localized prostate cancer.^{1, 2} One of the applications of such models allows for assessment of risk of disease recurrence after local therapy. However, levels of total PSA (tPSA) in blood do not solely refer to the presence of cancer, but are also driven by benign prostatic hyperplasia or inflammatory processes. Furthermore, Stamey and colleagues have shown that in men with moderately elevated levels of tPSA (<10 ng/ml), PSA does not aid in predicting biochemical cure rates.³ This limits the application of tPSA as a predictor of stage and disease progression in populations in which PSA is regularly used for screening. Hence, there's a compelling need for additional biochemical markers to improve detection of clinically significant, biologically aggressive prostate cancer.

One such marker is human kallikrein-related peptidase 2 (hK2), a product of the human KLK2 gene and a serine protease that shares 80% sequence homology with PSA (hK3). The enzymes share the property of being expressed chiefly in the prostate under androgen regulation.⁴ Recent studies point to substantial diagnostic capacity of molecular isoforms of PSA and hK2 for prostate cancer detection and disease staging.⁵ Furthermore, hK2 has been shown to provide independent prognostic information over other established preoperative parameters. Previous data demonstrated that increasing levels of hK2 and a low ratio of free PSA (fPSA) to tPSA are linked to advanced prostate pathology, which is a well-established risk factor for disease progression after surgery.^{6, 7} Subsequently, it was hypothesized that hK2 may be a useful biomarker especially for the detection of biologically aggressive prostate cancers.

Given the utility of current risk models to predict disease progression, rather than replacing these models, the aim of this study was to determine whether the predictive accuracy of standard preoperative risk models for BCR can be enhanced with the addition of molecular PSA isoforms (fPSA) and hK2.

MATERIALS AND METHODS

Patients and serum samples

Between January 1993 and December 2005, 4,737 patients with clinically localized prostate cancer (T1, T2, or T3 clinical tumor stage) underwent RP with staging lymphadenectomy at a single institution (Memorial Sloan-Kettering Cancer Center, New York, NY). Under an institutional review board–approved protocol investigating biochemical markers in prostate cancer, blood samples were obtained for 2,835 of these patients. Blood samples were drawn prior to RP 4 or more weeks after any prostatic manipulation (DRE, TRUS-guided biopsy), and immediately processed and frozen at –80°C until analysis. Patients with any neoadjuvant therapy ($n = 376$), prior surgical or medical treatment for benign prostatic hyperplasia ($n = 45$) or lack of follow-up information ($n = 1,294$) were omitted from this study, leaving 1,382 patients with corresponding blood samples eligible for analysis. There were 146 recurrences and the median follow-up for patients without recurrence was 3.2 years. The protocol was approved by the institutional review board and human tissue utilization committee, and informed consent had been obtained from all of the participating patients.

Clinical and pathologic evaluation

For each patient, the operating surgeon assigned a clinical stage according to the 1992 edition of the *AJCC Cancer Staging Manual*.^{8, 9} All RP specimens were surface-inked and processed using serial transverse sections at 3-mm intervals according to the Stanford protocol.¹⁰

Definition of biochemical recurrence

BCR was defined as postoperative levels of tPSA ≥ 0.40 ng/ml. The selection of this cut point was based on a previous evaluation, which demonstrated that a significant proportion of patients with a tPSA < 0.40 ng/ml did not experience further PSA rises.¹¹ None of the patients had received any adjuvant therapy before evidence of cancer recurrence.

Measurements of biomarkers

Total and free PSA—To measure tPSA and fPSA, we used the commercial version of a previously reported dual-label assay (DELFI A Prostat Dual Assay, PerkinElmer, Turku, Finland) that measures tPSA and fPSA on an equimolar basis.¹² Detection limits are 0.04 ng/ml for fPSA (coefficient of variation [CV] 3.7% at 0.44 ng/ml and 17.9% at 0.10 ng/ml), and 0.05 ng/ml for tPSA (CV 5.0% at 2.32 ng/ml and 13.9% at 0.34 ng/ml). The percentage of fPSA (%fPSA) was calculated as $\%fPSA = fPSA/tPSA \times 100$.¹³⁻¹⁵

Total hK2—Total hK2 was measured according to a previously reported three-step in-house assay design. It uses a combination of PSA-specific blocking monoclonal antibodies together with biotinylated capture F(ab')₂ and Europium-labeled monoclonal antibody detection recognizing both PSA and hK2. We used F(ab')₂ 6H10 as a capture antibody (which has $\approx 5\%$ cross-reaction to PSA) and Eu-labeled monoclonal antibody 7G1 as a tracer. Addition of PSA-specific blocking monoclonal antibodies (2E9, 5F7, and 5H6) enables specific detection of hK2 as it eliminates cross-reaction of PSA to $< 0.001\%$. Selective hK2 detection was performed by using F(ab')₂ 11B6 as a capture antibody, which is entirely specific for hK2, and Eu-labeled as a tracer. The functional detection limit is 0.003 ng/ml, defined as the concentration at which total assay imprecision ranged from 12.9% for low (0.0033 ng/ml) to 7.5% for high (0.48 ng/ml) hK2 levels.

Statistical methods

Free PSA and hK2 were not measured in a clinical setting, requiring frozen serum samples to obtain these measurements. In order to preserve frozen samples that need not be measured, we used a nested case-control study design matching on age and year of surgery. This design has been shown to be appropriate for determining associations and obtaining unbiased estimates of predictive accuracy.¹⁶ Therefore, the results currently presented would not importantly differ from that obtained from a Cox regression model. Cases were defined as patients with evidence of BCR at last follow-up. Three controls were randomly selected for each case from the patient population at risk at the time of BCR. The patient population at risk for a given case included all patients at risk (alive and without BCR) at the time at which the case recurred. By this definition, another case is a potential control if he had a longer time to biochemical recurrence. In other words, a patient who served as a control at a certain time point for a certain case could become a case once fulfilling the criteria for BCR himself. For the subgroup analysis of patients with moderately elevated tPSA levels, we re-matched within the patients with tPSA ≤ 10 ng/ml using the same methodology described above. The *sttocc* command in Stata was used to convert these survival-time data to case-control data.

Conditional logistic regression models were used to evaluate the association between predictors and BCR after RP. All biomarkers were entered as log-transformed continuous variables to model their non-linear association with outcome. The base multivariable model included log tPSA, clinical stage (categorized as $\leq T2a$ and $\geq T2b$), and biopsy Gleason grade (categorized as ≤ 6 , 3+4, 4+3, and ≥ 8). We then constructed multivariable models with log hK2 or log fPSA as additional predictors. Predictive accuracy was defined in terms of the area under the ROC curve (AUC). All AUCs were calculated using 10-fold cross-validation. Confidence intervals for the difference in AUC from the base model were obtained using bootstrap methods, resampling within case-control groups, with 1000 replications. Subgroup analyses focused on

men with moderately elevated PSA levels, who are more typical of contemporary patients with prostate cancer in the United States and in other countries, where tumors are generally detected by PSA screening.

The agreement between laboratory and clinical tPSA was assessed using the concordance correlation coefficient, which evaluates the degree to which pairs of observations are equivalent, and linear regression. All analyses were performed using Stata 9.2 (Stata, College Station, TX).

RESULTS

Overall, there were 146 cases with BCR and 436 controls (Supplement Table I). Of the 146 cases, 144 (99%) were matched to 3 controls. The remaining 2 cases (1%) were matched to only 2 controls because a total of 3 controls with matching age and year of surgery could not be found for them. Since age was a matching criterion, the age distribution between cases and controls was similar. Age was within 5 years of the matched case for all but 9 controls (2%). Year of surgery was matched exactly for all but 6 controls; these 6 controls varied from their case by 1 year of surgery. Clinical and pathological tumor characteristics were more unfavorable in cases than controls. For example, 63% of cases ($n = 92$) had a biopsy Gleason score >6 compared with only 23% of controls ($n = 100$); additionally, 57% of cases ($n = 83$) had advanced pathologic stage ($>pT2$) compared to only 22% of controls ($n = 96$).

Levels of tPSA, fPSA and hK2 are shown in Supplement Table II for the entire cohort and for patients with tPSA ≤ 10 ng/ml. Median levels of all biomarkers were higher in cases than controls. In the subgroup analyses for tPSA ≤ 10 ng/ml there were 70 cases and 209 controls. One case was matched to only 2 controls; the rest were matched to 3 controls. Overall, there were 34 patients who developed prostate cancer metastases, and 12 of them died of prostate cancer during the follow-up. However, as the numbers were small we did not perform detailed analyses for these outcomes.

In univariate analyses, all variables were significantly associated with BCR in the entire cohort. When applied to the subgroup of patients with tPSA ≤ 10 ng/ml, only clinical stage and biopsy Gleason score were statistically significant predictors of BCR (Table I). Previously established predictors (stage, grade, and tPSA) were combined in a prediction base model (Table II). The predictive accuracy of this model was high, with an AUC of 0.786 in the entire cohort. Adding fPSA and hK2 as predictors to the base model marginally enhanced the predictive accuracy to 0.798 in the entire cohort (difference in AUC 0.012; 95% confidence interval $-0.002, 0.032$). Of note, a statistically significant enhancement was observed when only fPSA was added to the base model (difference in AUC 0.013; 95% confidence interval 0.0004, 0.029). In the subgroup of men with tPSA ≤ 10 ng/ml, the addition of fPSA and hK2 enhanced the predictive accuracy of the base model to a similar degree (from 0.720 to 0.726; difference in AUC 0.006; 95% confidence interval $-0.019, 0.074$).

We also applied these analyses to a different prediction model, which also includes tPSA, but is mainly based on postoperative pathological parameters: RP specimen Gleason score, extracapsular extension, seminal vesical invasion, lymph node invasion, and surgical margin status (Supplement Table III). No enhancement in the predictive accuracy could be shown when fPSA or hK2, or both were added to this prediction model (Supplement Table IV).

Comparison of serum levels of tPSA between fresh and frozen samples

As described above, blood samples analyzed in this study were drawn prior to RP 4 or more weeks after any prostatic manipulation, and subsequently immediately processed, aliquotted, and frozen at -80°C until analysis in this study. At the same time, tPSA levels in serum were

determined in the clinical setting before patients underwent RP. We performed a subanalysis, in which we compared the concordance of levels of tPSA in both samples: (i) in the prospectively collected, aliquotted, and frozen samples that were then thawed and retrospectively measured (termed *laboratory* samples), and (ii) in the *clinical* samples that were analyzed for tPSA immediately on the day of venipuncture. The reason for this subanalysis was to address the question and hypothesis of possible biodegradation of PSA in the frozen *laboratory* samples. In a total of 427 patients, the date of the last clinical PSA measurement before RP and the date of the aliquot of the frozen *laboratory* sample were the same (i.e., the *fresh* sample, analyzed in the clinical setting, and the frozen sample, analyzed in this study, originated from the same initial venipuncture). Linear regression analysis demonstrated a statistically significant slope of 1.22, with an intercept of -0.49 , and $r^2 = 0.9540$, when tPSA values of *clinical* samples were compared to the corresponding *laboratory* samples (Supplement Figure 1A); the concordance correlation coefficient was 0.94. While the intercept of -0.49 showed the possibility of bias, this was highly influenced by 11 patients with outlying tPSA values (>30 ng/ml); when these patients were excluded from the linear regression, the slope was 1.16 and the intercept was -0.055 (Supplemental Figure 1B).

This subgroup of 427 patients with matching laboratory and clinical samples translated to 137 cases (94%) and 389 matched controls for the case-control analysis. The predictive accuracy of the base model for this subgroup of patients, when calculated with the tPSA values that were obtained from the *laboratory* samples, showed an AUC of 0.782, versus an AUC of 0.773 when calculated with tPSA levels from the *clinical* samples. Therefore, we can be confident that our conclusions were not affected by biodegradation of tPSA.

DISCUSSION

In this study we examined the ability of PSA isoforms (free PSA) and hK2 to improve upon previously validated risk-prediction models. Our findings suggest that an improvement in the AUC can be achieved by adding both fPSA and hK2 to the standard nomogram that contains only clinical stage, biopsy Gleason score, and tPSA.

Population-based data have shown that levels of PSA and hK2 are significantly elevated in blood up to 20 years before men were diagnosed with clinically significant prostate cancer.¹⁷ It is also believed that the tight compartmentalization of PSA and hK2 in the normal prostate gland is altered in prostatic disease. The disruption of the continuous layer of basal cells results in loss of the normal glandular architecture and allows substantial leakage of various proteins into circulation.^{18, 19} This development suggests that detection of increased extracellular kallikrein forms offer very sensitive means to detect changes in prostate tissue architecture that may signify prostate cancer progression.²⁰ The covariance of hK2 and PSA concentrations in blood has been determined to be generally $<60\%$, suggesting that hK2 might have utility as an independent biomarker for prostate cancer.²¹

Several studies have evaluated hK2 as a prognostic tumor marker. Darson and colleagues examined paraffin specimens from RP and found that hK2-specific immunostaining in lymphatic metastases and in high-grade tumors was more intense compared with well-differentiated tumors and benign tissues.²² Serum hK2 measurements were significantly elevated in locally advanced tumors compared with tumors that were organ confined.^{23, 24} Furthermore, on univariate analysis, hK2 alone was shown to be a significantly superior predictor of BCR compared with tPSA (concordance index of 0.739 for total hK2 vs. 0.566 for tPSA, $p < 0.0005$). Combining tPSA, fPSA, and total hK2 in a multivariable model improved prediction compared with use of each biomarker individually.⁷ We have previously reported that pretreatment measurements of hK2 and fPSA were helpful in identifying patients at risk for biochemical failure after RP.⁵ Overall, the predictive accuracy of the base model

(preoperative tPSA, clinical stage, and biopsy Gleason grade) was not improved after the addition of fPSA or hK2 (0.813 vs. 0.818). However, for men with moderate tPSA elevation (tPSA ≤ 10 ng/ml), addition of fPSA and hK2 data increased the predictive accuracy (from the base model concordance index of 0.756 to 0.815, $p = 0.005$).⁵ In our present study, while addition of fPSA and hK2 improved predictive accuracy for the entire cohort as well, the improvement was less robust (0.786 to 0.798). Additionally, in the cohort mentioned above,⁵ hK2, but not free PSA, was significantly associated with BCR on multivariable analysis in the subgroup of patients with a PSA of <10 ng/ml. In our cohort, we found the opposite: free PSA, but not hK2, was significantly associated with BCR. We believe that our results support the hypothesis that the combination of fPSA and hK2 (in addition to tPSA, stage, and grade) can enhance the predictive accuracy of BCR. We do not know, however, whether the main contribution to this enhancement is due to fPSA or hK2.

One of the critical aspects for patients and physicians is to better understand the risk a given prostate cancer poses on an individual basis. Currently available pretreatment staging tools can help in the decision-making process, but may lack specificity. We believe that the addition of new variables, such as serum peptide protein profiling, may improve our ability to better classify the biologic potential of individual prostatic tumors, and to distinguish between indolent and aggressive cancers. Improvement in the predictive accuracy may imply enhanced ability to counsel patients prior to treatment about their risk of BCR.

Several limitations may have influenced the validity of our findings. As suggested in multiple prior analyses on the value of free PSA and hK2, it is possible that our data may at least be partly influenced by suboptimal sample collection (we analyzed serum, not plasma), storage, and preanalytical processing, although the serum measurements were attempted to be made reliable and reproducible by following standard and precise processing protocols (separation of serum from blood cells immediately after venipuncture, subsequent immediate freezing at -80°C , analysis of samples immediately after thawing at 4°C), as well as limiting interference by replacing our monoclonal capture and tracer antibodies with recombinant Fab fragments.¹⁴ Hence, limited stability of free PSA and hK2 after venipuncture, reported also for long-term frozen serum samples as used in our analyses, instead of fresh plasma samples, may have influenced the accuracy of measurements of the analytes.^{25, 26} To address this question, we performed linear regression analysis for tPSA levels that were determined in the clinical setting, in comparison to the tPSA values in the frozen aliquots that were obtained through immunoassay measurements in this study. As mentioned, after excluding influential observations, this subanalysis demonstrated a statistically significant slope of 1.16, with an intercept of -0.055 . In other words, a man with a PSA of 1 ng/ml in the *clinical* sample would have a PSA of 0.95 ng/ml in the frozen *laboratory* sample, and then each additional 1-ng/ml increase in PSA level in a clinical sample would correspond to a 1.16-ng/ml increase in PSA level in the frozen *laboratory* sample.

Multiple factors have to be taken into consideration when trying to interpret these findings: First, biodegradation of the protein (PSA) itself could certainly, at least to a limited degree, contribute to a decrease in the measurable level of PSA in a frozen sample. Nevertheless, this process is very unlikely to be represented in such a linear manner as we were able to demonstrate in our analysis. Second, patient samples were collected from 1993 to 2005. During those years, important changes and developments with regard to immunoassay techniques that were used to detect PSA in serum or plasma had been made: First Assays that were used were less sensitive (i.e. detection limits of PSA were higher). But also simply interassay variability may be a factor that could explain the significant, but constant differences between PSA levels that were determined between the *clinical* samples and PSA levels that we measured in the aliquotted, frozen *laboratory* samples, which were analyzed much later and provided the data that went into the main analysis in this study. However, although the differences in the PSA

levels in serum were significant, we were able to show a linear relationship, which would preclude biodegradation in the frozen samples being the major reason for these differences. Our most important overall conclusion on this subanalysis is that PSA values obtained through measurements of the *laboratory* samples are in fact highly representative of the *clinical* values. This conclusion may seem a very important result of our analysis, as this finding confirms the biostability of tPSA samples that are stored and frozen for analyses at later time points.

In conclusion, our study replicated previous findings that serum hK2 and fPSA were associated with biochemical recurrence after local therapy. However, there was only a small increase in predictive accuracy when these markers were added to a standard multivariable model. This enhancement appeared to be largely contributed by fPSA. Given that fPSA is often routinely measured to aid detection of prostate cancer, incorporation in risk prediction models would be straightforward. Further studies are necessary to elucidate the role of these markers in predicting disease progression after local therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

National Cancer Institute; Grant number: P50-CA92629, SPORE Pilot Project 7; Grant sponsor: European Union 6th Framework; Grant number: LSHC-CT-2004–503011; Grant sponsor: Swedish Cancer Society; Grant number: 3555.

Abbreviations

AUC, area under the receiver operative characteristic curve; BCR, biochemical recurrence; CV, coefficient of variation; fPSA, free prostate-specific antigen; %fPSA, free-to-total prostate specific antigen ratio; hK2, human kallikrein 2; PSA, prostate-specific antigen; RP, radical prostatectomy; tPSA, total prostate-specific antigen.

References

1. Kattan MW, Eastham JA, Stapleton AM, Wheeler TM, Scardino PT. A preoperative nomogram for disease recurrence following radical prostatectomy for prostate cancer. *J Natl Cancer Inst* 1998;90:766–71. [PubMed: 9605647]
2. Partin AW, Kattan MW, Subong EN, Walsh PC, Wojno KJ, Oesterling JE, Scardino PT, Pearson JD. Combination of prostate-specific antigen, clinical stage, and Gleason score to predict pathological stage of localized prostate cancer. A multi-institutional update. *JAMA* 1997;277:1445–51. [PubMed: 9145716]
3. Stamey TA, Johnstone IM, McNeal JE, Lu AY, Yemoto CM. Preoperative serum prostate specific antigen levels between 2 and 22 ng./ml. correlate poorly with post-radical prostatectomy cancer morphology: prostate specific antigen cure rates appear constant between 2 and 9 ng./ml. *J Urol* 2002;167:103–11. [PubMed: 11743285]
4. Rittenhouse HG, Finlay JA, Mikolajczyk SD, Partin AW. Human Kallikrein 2 (hK2) and prostate-specific antigen (PSA): two closely related, but distinct, kallikreins in the prostate. *Crit Rev Clin Lab Sci* 1998;35:275–368. [PubMed: 9759557]
5. Steuber T, Vickers AJ, Haese A, Becker C, Pettersson K, Chun FK, Kattan MW, Eastham JA, Scardino PT, Huland H, Lilja H. Risk assessment for biochemical recurrence prior to radical prostatectomy: significant enhancement contributed by human glandular kallikrein 2 (hK2) and free prostate specific antigen (PSA) in men with moderate PSA-elevation in serum. *Int J Cancer* 2006;118:1234–40. [PubMed: 16152616]
6. Steuber T, Niemela P, Haese A, Pettersson K, Erbersdobler A, Felix Chun KH, Graefen M, Kattan MW, Huland H, Lilja H. Association of free-prostate specific antigen subfractions and human

- glandular kallikrein 2 with volume of benign and malignant prostatic tissue. *Prostate* 2005;63:13–8. [PubMed: 15378521]
7. Steuber T, Vickers AJ, Serio AM, Vaisanen V, Haese A, Pettersson K, Eastham JA, Scardino PT, Huland H, Lilja H. Comparison of free and total forms of serum human kallikrein 2 and prostate-specific antigen for prediction of locally advanced and recurrent prostate cancer. *Clin Chem* 2007;53:233–40. [PubMed: 17185368]
 8. Greene, FL.; Page, DL.; Fleming, ID.; Fritz, A.; Balch, CM.; Haller, DG.; Morrow, M. *AJCC Cancer Staging Manual*. Vol. 5th ed. Lippincott Raven Publishers; Philadelphia, PA: 1997.
 9. Sobin LH, Fleming ID. *TNM Classification of Malignant Tumors*, fifth edition (1997). Union Internationale Contre le Cancer and the American Joint Committee on Cancer. *Cancer* 1997;80:1803–4. [PubMed: 9351551]
 10. McNeal JE, Redwine EA, Freiha FS, Stamey TA. Zonal distribution of prostatic adenocarcinoma. Correlation with histologic pattern and direction of spread. *Am J Surg Pathol* 1988;12:897–906. [PubMed: 3202246]
 11. Amling CL, Bergstralh EJ, Blute ML, Slezak JM, Zincke H. Defining prostate specific antigen progression after radical prostatectomy: what is the most appropriate cut point? *J Urol* 2001;165:1146–51. [PubMed: 11257657]
 12. Mitrunen K, Pettersson K, Piironen T, Bjork T, Lilja H, Lovgren T. Dual-label one-step immunoassay for simultaneous measurement of free and total prostate-specific antigen concentrations and ratios in serum. *Clin Chem* 1995;41:1115–20. [PubMed: 7543033]
 13. Vaisanen V, Eriksson S, Ivaska KK, Lilja H, Nurmi M, Pettersson K. Development of sensitive immunoassays for free and total human glandular kallikrein 2. *Clin Chem* 2004;50:1607–17. [PubMed: 15247158]
 14. Vaisanen V, Peltola MT, Lilja H, Nurmi M, Pettersson K. Intact free prostate-specific antigen and free and total human glandular kallikrein 2. Elimination of assay interference by enzymatic digestion of antibodies to F(ab')₂ fragments. *Anal Chem* 2006;78:7809–15. [PubMed: 17105175]
 15. Vaisanen V, Pettersson K, Alanen K, Viitanen T, Nurmi M. Free and total human glandular kallikrein 2 in patients with prostate cancer. *Urology* 2006;68:219–25. [PubMed: 16844459]
 16. Pepe, M. *The Statistical Evaluation of Medical Tests for Classification and Prediction*. Oxford University Press; 2003. p. 25Oxford Statistical Science Series
 17. Lilja H, Ulmert D, Bjork T, Becker C, Serio AM, Nilsson JA, Abrahamsson PA, Vickers AJ, Berglund G. Long-term prediction of prostate cancer up to 25 years before diagnosis of prostate cancer using prostate kallikreins measured at age 44 to 50 years. *J Clin Oncol* 2007;25:431–6. [PubMed: 17264339]
 18. Lilja H. Biology of prostate-specific antigen. *Urology* 2003;62:27–33. [PubMed: 14607215]
 19. Savblom C, Malm J, Giwercman A, Nilsson JA, Berglund G, Lilja H. Blood levels of free-PSA but not complex-PSA significantly correlates to prostate release of PSA in semen in young men, while blood levels of complex-PSA, but not free-PSA increase with age. *Prostate* 2005;65:66–72. [PubMed: 15880475]
 20. Aus G, Becker C, Lilja H, Khatami A, Pihl CG, Hugosson J. Free-to-total prostate-specific antigen ratio as a predictor of non-organ-confined prostate cancer (stage pT3). *Scand J Urol Nephrol* 2003;37:466–70. [PubMed: 14675918]
 21. Becker C, Piironen T, Kiviniemi J, Lilja H, Pettersson K. Sensitive and specific immunodetection of human glandular kallikrein 2 in serum. *Clin Chem* 2000;46:198–206. [PubMed: 10657376]
 22. Darson MF, Pacelli A, Roche P, Rittenhouse HG, Wolfert RL, Saeid MS, Young CY, Klee GG, Tindall DJ, Bostwick DG. Human glandular kallikrein 2 expression in prostate adenocarcinoma and lymph node metastases. *Urology* 1999;53:939–44. [PubMed: 10223487]
 23. Haese A, Graefen M, Becker C, Noldus J, Katz J, Cagiannos I, Kattan M, Scardino PT, Huland E, Huland H, Lilja H. The role of human glandular kallikrein 2 for prediction of pathologically organ confined prostate cancer. *Prostate* 2003;54:181–6. [PubMed: 12518322]
 24. Haese A, Graefen M, Steuber T, Becker C, Pettersson K, Piironen T, Noldus J, Huland H, Lilja H. Human glandular kallikrein 2 levels in serum for discrimination of pathologically organ-confined from locally-advanced prostate cancer in total PSA-levels below 10 ng/ml. *Prostate* 2001;49:101–9. [PubMed: 11582588]

25. Piironen T, Pettersson K, Suonpaa M, Stenman UH, Oesterling JE, Lovgren T, Lilja H. In vitro stability of free prostate-specific antigen (PSA) and prostate-specific antigen (PSA) complexed to alpha 1-antichymotrypsin in blood samples. *Urology* 1996;48:81–7. [PubMed: 8973706]
26. Ulmert D, Becker C, Nilsson JA, Piironen T, Bjork T, Hugosson J, Berglund G, Lilja H. Reproducibility and accuracy of measurements of free and total prostate-specific antigen in serum vs plasma after long-term storage at –20 degrees C. *Clin Chem* 2006;52:235–9. [PubMed: 16384894]
27. Pepe, MS. *The Statistical Evaluation of Medical Tests for Classification and Prediction*. Oxford University Press; Oxford, England: 2003. Oxford Statistical Science Series (31)
28. Hanley JA, Hajian-Tilaki KO. Sampling variability of nonparametric estimates of the areas under receiver operating characteristic curves: an update. *Acad Radiol* 1997;4:49–58. [PubMed: 9040870]

Univariate and multivariable results of conditional logistic regression for prediction of biochemical recurrence following radical prostatectomy among all patients and among patients with total PSA ≤ 10 ng/ml, controlling for clinical tumor characteristics

	Univariate			Multivariable, full model		
	Odds ratio	95% CI	<i>p</i> value	Odds ratio	95% CI	<i>p</i> value
<i>Cases = 146, controls = 436</i>						
Clinical stage						
T1c/T2a (reference)	—	—		—	—	
T2b/c/T3	2.27	1.53, 3.37	<0.0005	1.34	0.80, 2.26	0.3
Biopsy Gleason score						
≤6 (reference)	—	—		—	—	
3+4	1.49	0.92, 2.07		—	—	
4+3	2.74	1.97, 3.51		4.46	2.36, 8.44	
≥8	2.49	1.71, 3.26		13.2	5.56, 31.3	
log total PSA	2.75	2.04, 3.73	<0.0005	11.61	4.89, 27.6	<0.0005
log free PSA	1.42	1.09, 1.85	0.009	4.49	2.63, 7.67	0.001
log hK2	1.74	1.37, 2.21	<0.0005	0.43	0.26, 0.71	0.3
				1.20	0.87, 1.66	
<i>PSA ≤10 ng/ml cases = 70, controls = 209</i>						
Clinical stage						
T1c/T2a (reference)	—	—	0.005	—	—	0.3
T2b/c/T3	2.17	1.26, 3.72		1.47	0.76, 2.85	
Biopsy Gleason score						
≤6 (reference)	—	—		—	—	
3+4	0.64	−0.20, 1.47		—	—	
4+3	2.69	1.48, 3.90		1.84	0.78, 4.35	
≥8	2.39	1.33, 3.44		11.44	3.25, 40.3	
log total PSA	1.45	0.86, 2.47	0.17	10.18	3.27, 31.7	0.02
log free PSA	0.85	0.56, 1.28	0.4	2.6	1.14, 5.92	0.07
log hK2	1.12	0.82, 1.52	0.5	0.51	0.25, 1.06	0.6
				1.09	0.74, 1.61	

Table II

Accuracy (defined in terms of the area under the ROC curve (AUC)) of a preoperative prediction base model (including clinical stage, biopsy Gleason score, and total PSA), compared to the base model supplemented by free PSA and/or hK2 levels

	AUC	95% CI for difference from base	P value for difference from base
<i>Cases = 146, controls = 436</i>			
Base model	0.786		
+ free PSA	0.799	0.0004, 0.029	0.017
+ hK2	0.783	-0.006, 0.007	0.8
+ free PSA & hK2	0.798	-0.002, 0.032	0.053
<i>tPSA ≤10 ng/ml cases = 70, controls = 209</i>			
Base model	0.720		
+ free PSA	0.737	-0.010, 0.072	0.13
+ hK2	0.707	-0.021, 0.035	0.7
+ free PSA & hK2	0.726	-0.019, 0.074	0.2