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Diagnostická a prognostická schopnost vybraných markerů karcinomu prostaty v séru a moči

Diagnostic and prognostic ability of selected markers of Prostate cancer in the serum and urine

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Diagnostická a prognostická schopnost vybraných markerů karcinomu prostaty v séru a moči

Abstrakt

Sérový prostatický specifický antigen (PSA) je jediným široce schváleným markerem v diagnostice a sledování rakoviny prostaty (PC) po léčbě. Jeho role zůstala kontroverzní kvůli nedostatečné specifitě a riziku nadměrné diagnózy nevýznamného PC. Cílem této práce bylo prozkoumat slibné markery PC a zlepšit současnou stratifikaci pacientů k adjuvantní léčbě. Byly provedeny tři hlavní studie s použitím různých médií (moč a sérum). První studie zahrnovala hodnocení Engrailed-2 (EN2) – sledovaného močového markeru – u 90 pacientů s lokalizovaným PC, 30 zdravých kontrol a 40 pacientů indikovaných k biopsii prostaty. Druhá studie hodnotila 205 mužů s vysoce rizikovými rysy PC, kteří podstoupili radikální prostatektomii (RP) a byli podrobeni přísnému protokolu sledování ultrasenzitivního PSA (UPSA) v krátkých časových intervalech. Schopnost jednotlivých měření predikovat biochemickou recidivu (BCR) a tím nutnost adjuvantní terapie byla hodnocena pomocí plochy pod křivkou (AUC) a byl vytvořen stratifikační model. Třetí studie zahrnovala 128 pacientů, kteří podstoupili RP. PSA a jeho sérové izoformy běžně používané v diagnostickém kontextu byly hodnoceny předoperačně i pooperačně, aby se určila jejich schopnost predikovat BCR. Analýza EN2 v moči nemá klinickou užitečnost při detekci PC. UPSA již 30. den po RP je dobrým prediktorem BCR u mužů s nepříznivými patologickými rysy a může snížit přeléčení adjuvantní radioterapií. Sérové PHI a [–2]proPSA překonávají sérové PSA v predikci BCR a jejich použití v klinických predikčních modelech a nomogramech by mělo velkou hodnotu. Izoformy PSA nemají žádnou roli ve sledování pacientů s PC po RP.

Klíčová slova: Karcinom prostaty, PSA, biomarkery, izoformy PSA, ultrasenzitivní PSA, [–2]proPSA, PHI, hK2, EN2

The diagnostic and prognostic ability of selected serum and urinary markers of prostate cancer

Abstract

Serum prostate specific antigen (PSA) is the only widely approved marker in prostate cancer (PC) diagnosis and follow up. Its role has remained controversial due to lack of specificity and the risk of overdiagnosis of insignificant PC. The aim of this work was to explore promising markers of PC and to improve current patient stratification to adjuvant treatment. Three main studies were performed using different media (urine and serum). The first study included the evaluation of Engrailed-2 (EN2) – a urinary marker of interest – in 90 patients with localized PC, 30 healthy controls, and 40 patients indicated for prostate biopsy. The second study evaluated 205 men with high-risk PC-features who underwent radical prostatectomy (RP) and were subject to a follow-up of ultrasensitive PSA (UPSA) at close time intervals. The ability to predict biochemical recurrence (BCR) and thus the need for adjuvant therapy was assessed using the area under the curve (AUC) and a stratification model was created. The third study involved 128 patients who underwent RP. PSA and its serum isoforms normally used in the diagnostic context were evaluated both preoperatively and postoperatively to determine their ability to predict BCR. Analysis of EN2 in the urine has no clinical usefulness in the detection of PC. UPSA as early as day 30 after RP is a good predictor of BCR in men with adverse pathological features and can decrease overtreatment with adjuvant radiotherapy. Serum PHI and [–2]proPSA outperform serum PSA in the prediction of BCR and their use in clinical prediction models and nomograms would be of great value. There is no role for PSA isoforms in the follow up of PC patients after RP.

Keywords: Prostate cancer, PSA, biomarkers, PSA isoforms, ultrasensitive PSA, [–2]proPSA, PHI, hK2, EN2

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1. Introduction

Prostate cancer (PC) is currently the third most common diagnosed malignancy, being preceded by lung and colorectal cancer with incidence rates across the world ranging from 6.3 to 83.4/100.000 (1). Only three risk factors for PC are defined: age, race (African) and positive family history (association of BRCA1 and BRCA 2 mutations) (2). PC diagnosis is currently done by prostatic specific antigen (PSA) testing, digital rectal examination (DRE) and if the suspicion in the previous two is present, prostate biopsy. The disease is graded according to a two-grade system called Gleason score and the total Gleason score ranges from 6 (least aggressive) till 10 (most aggressive). More recently in 2014 the International Society of Urological Pathology (ISUP) issued a new grading system (1-5) (3). Disease staging is according to the 2018 Clinical Tumor Node Metastasis (TNM) classification (4). Mass screening with PSA has been controversial (5) over the years due to lack of marker specificity (6) (7), substantial overdiagnosis of indolent cases (8), disease overtreatment and doubts on the decrease of disease specific mortality even with extended follow-up (9) (10). The present recommendation (11) is to offer a PSA test to well informed men with a life expectancy of 10-15 years starting at the age 50 years or 45 years in case risk factors are present. A PSA between 3-10 ng/ml should prompt a repeat analysis 4-7 weeks later. For a cut-off of 4ng/ml PSA has sensitivity of 67.5-80% (12) (13) and specificity of 60-70% (13). Age-adjusted values, the ratio of free PSA (FPSA) to total PSA and PSA density (ng/ml/cc) can be used to increase the otherwise low specificity. The current recommendation (11) for men with a normal DRE and a PSA between 3-10 ng/ml is to perform a reflex test before prostate biopsy (a risk calculator, magnetic resonance or a marker test). Both 4K score and the Prostate health index (PHI) formula using [–2]proPSA (a precursor of PSA) are approved for use at decision for both

biopsy-naïve patients and at repeat biopsy. Among other approved marker tests are IsoPSA (it detects structural isoforms of PSA with an aqueous 2-phase system), urine PCA3 (decision to repeat a biopsy) and SelectMDX (urine molecular test that combines the expression of mRNA of genes of interest with clinical risk factors). Regarding imaging methods, transrectal ultrasound is used to guide prostate biopsies but it is not reliable to detect PC, and multiparametric magnetic resonance is the exam of choice when there is a suspicion based on PSA and/or digital rectal examination. It should be performed before prostate biopsy and it classifies lesions attributing a score from 1-5 (PI-RADS™v2 classification) with lesions ≥3 indicated for target biopsy-only in cases of a repeat biopsy or together with systematic sampling in biopsy-naïve patients. A minimum of 8 cores in a 30cc prostate and 10-12 cores in larger size prostates according to a scheme is the current recommendation (11). Prostate biopsy can be performed via the transrectal or transperineal route (less infectious complications, less bleeding, increased detection of anterior zone tumors). After obtaining histological confirmation, disease staging should be managed in the following way: intermediate (PSA 10-20 ng/ml or ISUP2/3 or cT2b) and high-risk disease (PSA>20ng/ml or ISUP 4/5 or ≥cT2c or cN+) should be staged with an abdominopelvic cross-sectional examination (CT or MRI) and a 99mTc-Bone bone scan. Low risk disease does not require any staging besides clinical staging with DRE performed in all prostate cancer patients.

In patients with localized disease a life expectancy of minimum 10 years is mandatory for a benefit of active treatment. Patients with a life expectancy less than 10 years or unsuitable for curative treatment due to comorbidities are clinically watched for the development of symptoms (due to local or systemic progression) and managed palliatively at that time. Patients with low-risk disease can be managed with active surveillance (PSA samples, DRE, MRI imaging and repeat biopsies) or active treatment (radical prostatectomy,

radiotherapy, brachytherapy). Intermediate and high-risk patients can be managed by radical prostatectomy (RP), radiotherapy combined with androgen deprivation therapy (ADT) or a combination of brachytherapy, radiotherapy and ADT.

Preoperative nomograms exist such as Partin tables (14) (15), MSKCC nomogram (16) and the Briganti nomogram (17) to help predict organ-confined disease, seminal vesicle involvement, extraprostatic extension and lymph node invasion. Decision to perform lymph node dissection is based on these nomograms.

Treatment of locally advanced disease can include RP with pelvic lymph node dissection as part or multimodal therapy followed by radiotherapy or radiotherapy with/without a brachytherapy boost with long term ADT.

Adjuvant treatments are defined as additional to the initial therapy with the objective of reducing the risk of biochemical recurrence (BCR). The current indications (11) for adjuvant treatment are patients with final pathology at radical prostatectomy ISUP 4-5 and pathological stage pT3 with or without positive surgical margins. For patients with positive lymph nodes at final pathology the options are adjuvant ADT with/without radiotherapy or observation in case the number of nodes is <2 and the PSA < 0.1 ng/mL.

After primary therapy patients are subject to PSA monitoring at regular intervals. After RP the definition of BCR is a detectable or rising PSA ≥ 0.2ng/ml with a second confirmatory level ≥ 0.2ng/ml. After radiotherapy, treatment failure is defined by the Phoenix criteria as an increase in PSA of at least 2ng/ml above the post radiation PSA nadir. The relevance of BCR is that it precedes metastases with a mean time of 8 years and specific mortality of about 15 years (18). Persistent PSA is defined as a PSA level ≥0.1 ng/ml 4-8 weeks after RP. It can result from persistent disease, pre-existing metastases, lymph node involvement or residual benign tissue and the current treatment is

early salvage radiotherapy and additional hormonal therapy. Salvage treatments are indicated when BCR occurs. Radiotherapy combined with ADT is indicated if surgery was performed before and in case radiotherapy was the primary treatment, monitoring, salvage local procedures including surgery or ADT are considered.

Around 20% of men have urinary incontinence 12 months after RP and most patients (78-87%) are affected by erectile dysfunction (19). Late adverse events after radiotherapy include mainly genitourinary and gastrointestinal toxicity. Median rates of moderate late toxicity are 15% (gastrointestinal) and 17% (genitourinary). For severe toxicity these values were 2% (gastrointestinal) and 3% (genitourinary) (20).

Treatment of M1 patients is constantly evolving and ADT plus an additional systemic therapy such as chemotherapy or second-generation hormonal therapy with Abiraterone, Enzalutamide or Apalutamide is currently recommended. In cases of low-volume M1 disease, prostate radiotherapy in addition to ADT can be beneficial.

2. Aims of the work and hypothesis

Our work aims at studying new forms of improving PC diagnosis and prognosis and analyzing early PSA kinetics after RP to decrease overtreatment with adjuvant radiotherapy.

2.1. Study 1: The role of Engrailed 2 in prostate cancer detection

Urinary Engrailed-2 (EN2) is a promising albeit understudied marker of PC. Its features include exclusive secretion by prostate cancer cells, association to tumor volume and stage, simple sampling and easy non-invasive collection. The aim of this work was to test this marker in a comprehensive manner including a control group, multi-brand assays including previous prostate

manipulation. Hypothesis: EN2 can be used in diagnosis of PC adding important information regarding tumor size and stage and prostatic manipulation will increase its urinary levels.

2.2. Study 2: Stratification model based on early postprostatectomy prostate-specific antigen kinetics may help to reduce the risk of overtreatment in candidates for adjuvant radiotherapy

There is significant heterogeneity in the group of patients at high-risk for BCR after RP and nonetheless an 'adjuvant treatment to all' approach with radiotherapy is used. This leads to potentially avoidable adverse events. The aim of this study was to create a model to better stratify which patients benefit from adjuvant treatment based on multiple PSA analysis in the early period after surgery. Hypothesis: Early and multiple PSA sampling after RP will provide an optimal timing and cut-off value and allow stratification of patients for adjuvant treatment.

2.3. Study 3: Early prediction of prostate cancer biochemical recurrence and identification of disease persistence using PSA isoforms and human kallikrein-2

There has been great interest in the use of isoforms of PSA such as [–2]proPSA, PHI and 4K panel in the diagnostic setting of PC although studies as preoperative predictors of BCR are still scarce. PSA is currently the only parameter used in preoperative predictive nomograms and the only tool used in disease follow up after primary treatment. The aim of this work was to study the role of PSA isoforms and human kallikrein-2 in the pre- and early postoperative period in predicting and assessing BCR and disease persistence. Hypothesis: Isoforms of PSA outperform conventional PSA in predicting BCR and disease persistence.

3. Materials and methods

3.1. Study 1: The role of Engrailed 2 in prostate cancer detection

Morning urine samples of two groups were analyzed. The first group consisted of 90 patients with clinically localized PC before RP and a control group of 30 healthy men older than 50 years of age with a negative oncologic history and screening. The second group included 40 patients indicated for prostate biopsy. In the latter group pre- and post-DRE urine samples were obtained. Urine samples were stored in 1.8ml aliquots at -76ºC and analyzed using 3 different enzyme-linked immunoassays (Cloud-Clone Corp, Katy, TX, USA; Cusabio Biotech Co., LTD., Houston, TX, USA and MyBiosource, Inc., San Diego, CA, USA). Measurements were performed in a blind manner by a single operator and standardization was assured by the creation of a calibration curve. A four-parameter logistic curve was used for calculation of the EN2 concentration. Normalization to urinary creatinine (assessed by ADVIA Siemens and Orto Vitro) was additionally performed. Correlations were determined using Spearman's rank correlation coefficient. The Mann–Whitney U test was used to compare the patient group with the control group, while the Kruskal–Wallis H test was used to compare the EN2 distributions of the three different assays. Receiver operating characteristic (ROC) curves were built to evaluate the area under the curve (AUC) of urinary EN2 obtained with the different assays and serum PSA. p values <0.05 were considered significant.

3.2. Study 2: Stratification model based on early postprostatectomy prostate-specific antigen kinetics may help to reduce the risk of overtreatment in candidates for adjuvant radiotherapy

A total of 406 patients from department's tumor registry with a minimum follow up of 24 months who had adverse pathology after radical

prostatectomy (positive surgical margins, extracapsular extension and/or seminal vesicle invasion) were included in the study. Patients who had neoadjuvant or adjuvant forms of therapy (radiotherapy or hormonal therapy), missing follow up data, positive lymph nodes or a postoperative nadir > 0.1ng/ml were excluded. A final group of 205 patients was available for statistical analysis. Serum PSA analyses were carried out (IMMULITE 2000 3rd Generation PSA; Siemens Medical, Los Angeles, CA) postoperatively on days 14, 30, 60 and 90 and at 3-month intervals thereafter. BCR was defined as PSA persistently greater than or equal to 0.2 ng/ml. Variables between groups of patients were compared with the Mann–Whitney test and chi squared test. Recurrence-free survival curves were calculated using the Kaplan-Meier estimator with significance evaluated by the stratified log-rank test. Multivariate analysis was performed using the Cox proportional hazards model. The ROC curve and AUC were calculated to describe the accuracy of PSA measurements in predicting BCR after surgery. A mathematical model was built to provide a sequential algorithm to select patients for early intervention. A p value less than or equal to 0.01 was considered statistically significant. Statistical analysis was performed using the SAS statistical software program JMP 6 (SAS Institute, Cary, NC) and R statistical software version 3.1.2 (R Foundation for Statistical Computing, Vienna, Austria).

3.3. Study 3: Early prediction of prostate cancer biochemical recurrence and identification of disease persistence using PSA isoforms and human kallikrein-2

A group of 128 consecutive patients who underwent open or laparoscopic RP for clinically localized PC was studied. Blood samples were collected preoperatively and postoperatively at 1 and 3months after surgery without previous prostatic manipulation. When BCR or disease persistence occurred, patient follow up was ended. No patient received preoperative or

postoperative ADT or adjuvant radiotherapy (ART). The median (range) follow up period was 64 months (3–76 months). A total of 87 patients were BCR-free while 26 patients had BCR (20.3%) and 15 patients had disease persistence (11.7%). All blood samples were centrifuged for 15 minutes at 3000 revolutions per minute, the serum pipetted into 1,8ml aliquots (Nunc Cryotube Vials, Thermo Fisher scientific, Roskilde, Denmark) and stored at –76◦C. The markers assessed were PSA, fPSA, [–2]proPSA, PHI and hk2. BCR was defined as 2 consecutive rises of PSA > 0.2 ng/ml, while disease persistence was defined as PSA ≥ 0.1 ng/ml at 6 weeks after radical prostatectomy. Variables of interest were compared between studied groups of patients and the BCR-free group using the Mann-Whitney Wilcoxon U-test in case of numerical variables and the Fisher Exact test in case of categorical variables. Multivariable models were constructed using logistic regression. To assess the predictive value of the variables ROC analysis including an AUC evaluation was adopted. Statistical analyses were performed using an R statistical package version 3.6.3. and XLSTAT version 2020.1 (Addinsoft Inc., New York, United States). P-values <0.05 were considered statistically significant.

4. Results

4.1. Study 1: The role of Engrailed 2 in prostate cancer detection

4.1.1. Urinary EN2 in patients with prostate cancer versus controls

There was no statistically significant difference between the EN2 urinary levels of the patient and the control group and there was a pronounced difference between the EN2 levels measured by the three assays (Kruskal–Wallis p-value <0.0001). Normalization of the EN2 level to urinary creatinine showed similar statistical distributions. Analysis of ROC curves and

calculation of AUC values showed that urine EN2 levels did not reach the predictive accuracy of conventional PSA (AUC=0.70) (Fig. 1). Furthermore, no significant correlation between urinary EN2 and age, tumor stage or grade was found.

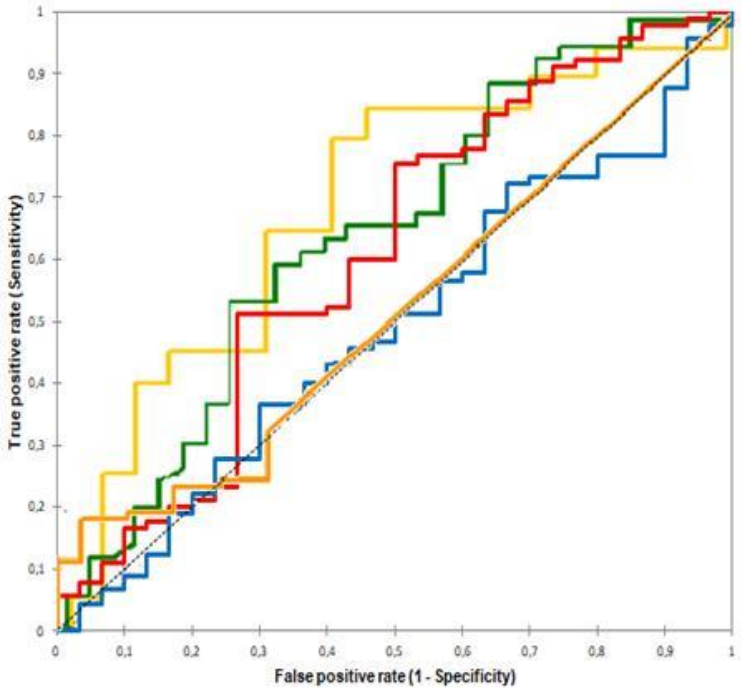

Fig 1: Comparison of the ROC curves obtained for EN2 measured with kits 1, 2 and 3 and the ROC curve for PSA. Red: ROC curve for EN2-kit1, AUC= 0,61, Blue: ROC curve for EN2/urine Creatinine-kit1, AUC =0,49, Orange: ROC curve for EN2-kit2, AUC= 0,52, Green: ROC curve for EN2-kit3, AUC=0,65, Yellow: ROC curve for PSA, AUC=0,70

4.1.2. Urinary EN2 pre- and post-digital rectal examination in patients indicated for prostate biopsy

The levels of EN2 (Fig. 2) were lower after DRE (median 1.79 ng/ml; range 0.12–5.01 ng/ml) compared to before (median 2.29 ng/ml; range 0.22–5.31 ng/ml) with a p-value for these two groups of 0.18. In the biopsy-negative patients (n=20) EN2 changed from 2.38 ng/ml (0.41–5.31 ng/ml) to 1.99 ng/ml (0.64–5.01 ng/ml) after DRE while in the biopsy-positive group (n=20) the median (range) of EN2 changed from 2.21 ng/ml (0.22–3.33 ng/ml) before DRE to 1.51 ng/ml (0.12–3.55 ng/ml). The p-values obtained for the two

groups were 0.30 (before DRE) and 0.18 (after DRE). Normalization to urine creatinine did not change the statistical distributions significantly.

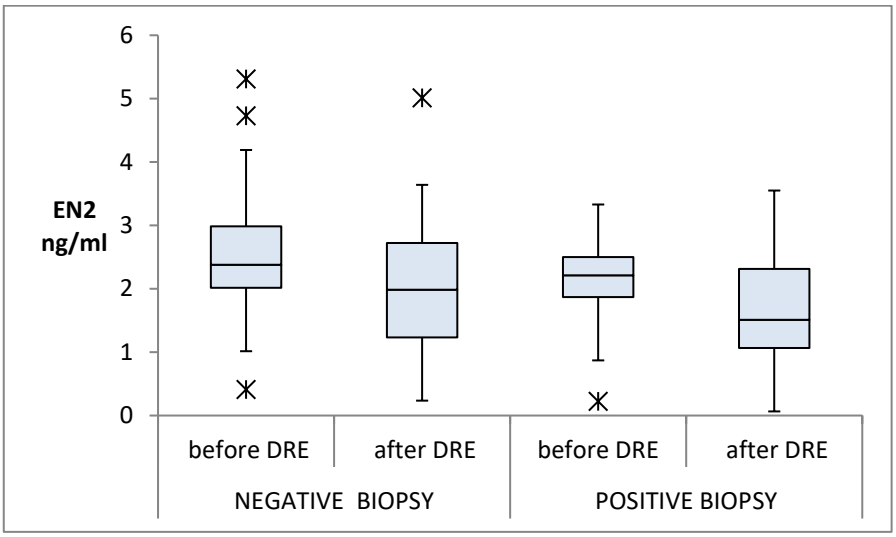

Fig. 2: EN2 concentrations in urine before and after digital rectal examination

4.2. Study 2: Stratification model based on early postprostatectomy prostate-specific antigen kinetics may help to reduce the risk of overtreatment in candidates for adjuvant radiotherapy

Extraprostatic extension was present in 113 men (55%), positive surgical margins were present in 97 men (47%) and seminal vesicle invasion was present in 32 men (16%) During the median follow-up of 46 months (range 24–114 months), a total of 106 patients (52%) experienced BCR. The median time to recurrence was 15 months (range 2–105 months). Only five men had the combination of all the previously stated adverse pathological features and these patients did not experience a significantly different rate of recurrence (60%) in comparison with the rest of the cohort (51.5%, p=0.707). A similar frequency of recurrence (54%) was found in men with a Gleason score higher than 7 together with positive surgical margins or pT3. Median PSA values for patients with BCR and without BCR on days 14, 30, 60, 90 and 180 were 0.286 ng/ml and 0.204 ng/ml ($p<0.02$), 0.060 ng/ml and 0.025 ng/ml ($p<0.0001$), 0.026 ng/ml and 0.009 ng/ml ($p<0.0001$), 0.036 ng/ml and 0.007 ng/ml ($p<0.0001$), and 0.049 ng/ml and 0.009 ng/ml ($p<0.0001$), respectively.

Fig. 3 shows the ROC curves used in prediction of BCR at different time points.

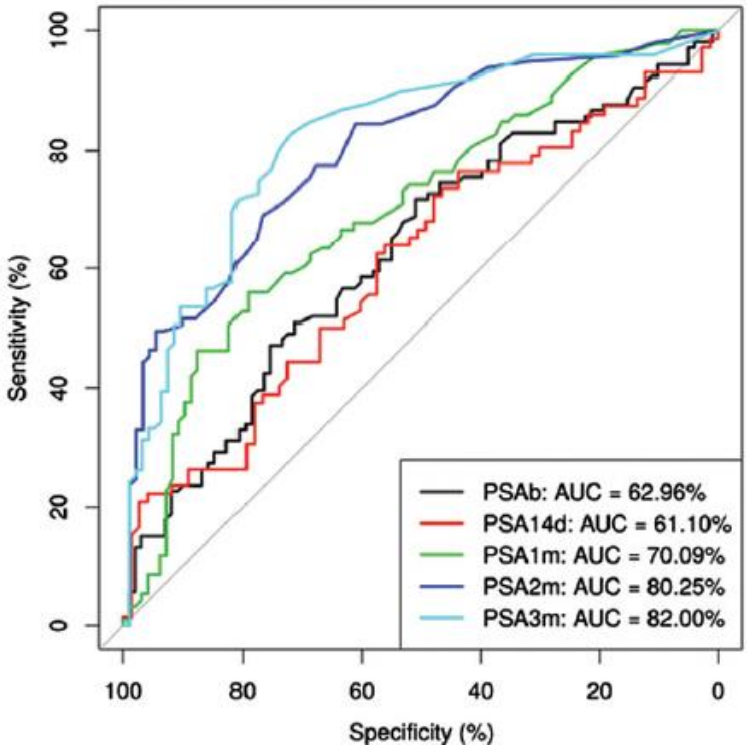

Fig. 3: ROC curves and calculated AUC values for preoperative (baseline) prostate-specific antigen (PSAb) level and postoperative PSA levels at time-points after surgery: 14 days, and 1, 2 and 3 months.

As can be seen in Fig. 3 baseline preoperative PSA and PSA at day 14 did not significantly predict BCR and the prediction ability increased gradually with time since surgery. The improvement in the predictive ability was significant between PSA on days 14 and 30 (p=0.01) and between days 30 and 60 (p=0.042) while further improvement between days 60 and 90 was not significant (p=0.694). The first valuable prediction of BCR is possible at day 30, while the accuracy increases to 80% on day 60 after surgery. Therefore, PSA levels on days 30 and 60 were used in the construction of a sequential decision model to select the best candidates for early intervention. The final stratification model with the best predictive accuracy (AUC=0.76) resulted in PSA cut-offs of 0.068 ng/ml and 0.015 ng/ml for days 30 and 60, respectively (Fig. 4).

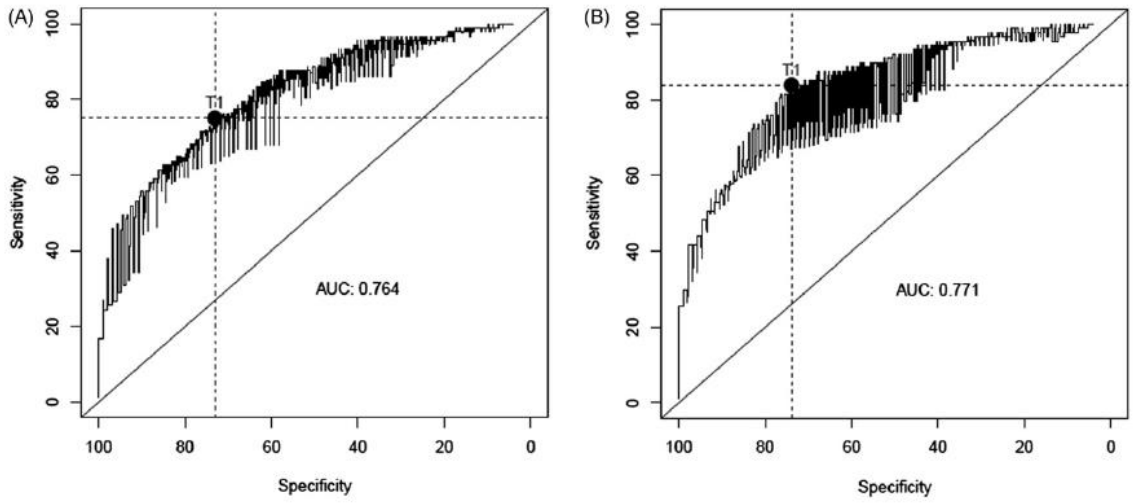

Fig. 4: Results of the sequential decision calculating the best combination of postoperative PSA cut-off levels in the model combining the PSA (A) on days 30 and 60, and (B) on days 30 and 90.

In this study, out of 172 patients, 51% did not develop BCR during follow up and this indicates the proportion of potential overtreatment. Patients (n=52) presenting with PSA levels over 0.068 ng/ml on day 30 would be indicated directly for early postoperative ART. The other 120 patients would continue to PSA measurement on day 60. Those (n=35) with PSA above 0.015 ng/ml on day 60 would be again indicated for ART. The rest of the patients would continue with routine follow-up. Applying this stratification model would result in a decrease of overtreatment from the initial 51% (n=87) to 14% (n=24). Of the 22 patients who would stay undertreated, 18 patients would reveal the PSA progression on day 90 and another two patients on day 120, while only two patients would stay undertreated, with the late appearance of BCR after 39 and 48 months.

4.3. Study 3: Early prediction of prostate cancer biochemical recurrence and identification of disease persistence using PSA isoforms and human kallikrein-2

PSA, fPSA, %fPSA, [–2]proPSA, PHI and hK2 were evaluated before surgery, at 1 and 3 months after surgery. A total of 87 patients were recurrence-free while 26 patients had BCR (20.3%) and 15 patients had

disease persistence (11.7%). In the preoperative setting, the ability of PSA to predict recurrence (AUC 0.64; p-value 0.029) was surpassed by [–2]proPSA (AUC 0.70; p-value 0.002) and, more importantly, PHI (AUC 0.73; p-value 0.0003) (Fig. 5).

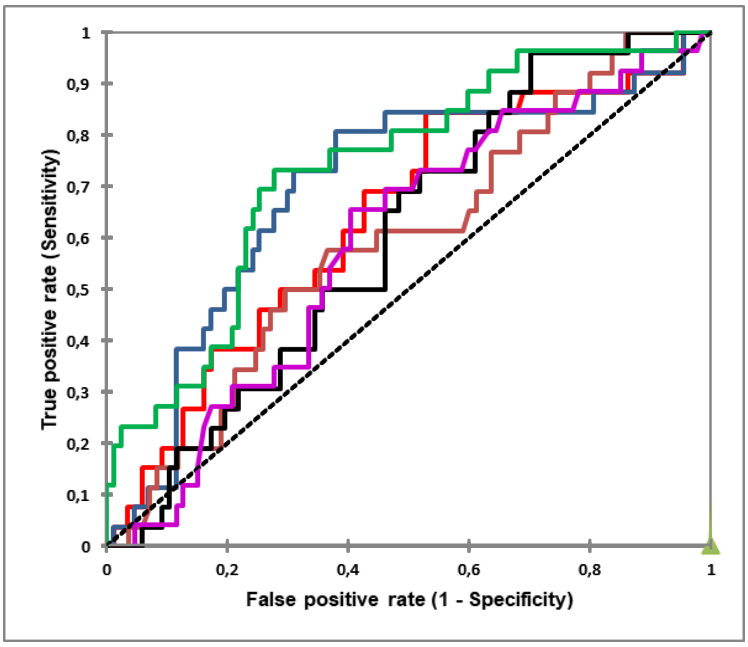

Fig 5: ROC curves for all the markers at preoperative period and their relation to BCR. Red PSA(AUC0.64), Blue [–2]proPSA (AUC 0.70), Green PHI (AUC 0.74), Brown hK2 (AUC 0.60), Violet fPSA (AUC 0.59), Black fPSA/PSA (AUC 0.60).

In the preoperative period [–2]proPSA (AUC 0.73; p-value 0.0055) and PHI (AUC 0.75; p-value 0,0021) also outperformed PSA (AUC 0.68; p-value 0.026) in predicting disease persistence. In the postoperative period, PSA was the only marker that correlated with BCR at one (AUC 0.69) and three months (AUC 0.72) and all other markers were devoid of value. Multivariate models using preoperative data were created (Tab. 1) and confirmed the superiority of preoperative PHI in predicting disease relapse both when used alone or when combined to PSA (AUC 0.86; *p*-value <0.0001).

Table 1: Multivariate models using preoperative data and selected markers

Model	Preoperative Marker	Preoperative parameters	Multivariate analysis	Model performance
Model A	PSA	Clinical stage	OR 1.8967 (0.8597 – 4.1845) p-value ≥ 0.05	AUC 0.8013 (0.7081 – 0.8945) p-value < 0.0001
		Preoperative Gleason score	OR 4.1163 (2.0283 – 8.3538) p-value 0.0001	
		Preoperative PSA	OR 1.0652 (0.9789 – 1.159) p-value ≥ 0.05	
Model B	[−2]proPSA	Clinical stage	OR 1.8841 (0.8491 – 4.1809) p-value ≥ 0.05	AUC 0.8139 (0.7215 – 0.9063) p-value < 0.0001
		Preoperative Gleason score	OR 4.1587 (2.0359 – 8.4948) p-value 0.0001	
		Preoperative [−2]proPSA	OR 1.0272 (0.9988 – 1.0565) p-value ≥ 0.05	
Model C	PHI	Clinical stage	OR 2.167 (0.9111 – 5.1541) p-value ≥ 0.05	AUC 0.8616 (0.7853 – 0.9380) p-value < 0.0001
		Preoperative Gleason score	OR 4.5826 (2.1087 – 9.959) p-value 0.0001	
		Preoperative PHI	OR 1.0308 (1.0132 – 1.0487) p-value < 0.001	
Model D	PSA [−2]proPSA	Clinical stage	OR 1.8864 (0.8502 – 4.1853) p-value ≥ 0.05	AUC 0.8152 (0.7248 – 0.9056) p-value < 0.0001
		Preoperative Gleason score	OR 4.1448 (2.0254 – 8.4819) p-value 0.0001	
		Preoperative PSA	OR 1.0078 (0.8886 – 1.143) p-value ≥ 0.05	
		Preoperative [−2]proPSA	OR 1.0252 (0.9832 – 1.0691) p-value ≥ 0.05	
Model E	PSA PHI	Clinical stage	OR 2.1729 (0.9147 – 5.162) p-value ≥ 0.05	AUC 0.8634 (0.7866 – 0.9402) p-value < 0.0001
		Preoperative Gleason score	OR 4.7032 (2.1549 – 10.2652) p-value 0.0001	
		Preoperative PSA	OR 0.9503 (0.8478 – 1.0653) p-value ≥ 0.05	
		Preoperative PHI	OR 1.0367 (1.0136 – 1.0604) p-value 0.0017	

When analyzing predictors of disease persistence, PSA (AUC 0.68; p-value 0.026), fPSA (AUC 0.66; p-value 0.048), PHI (AUC 0.75; p-value 0.002) and [–2]proPSA (AUC 0.73; p-value 0.006) were found to be significant at the preoperative period while in the postoperative period PSA remained the marker of choice.

5. Discussion

Our results on urinary EN2 oppose those of Morgan et al. (the first experimental work on EN2 as a marker of PC) published in 2011 (21) and those of Pandha et al (22) published the following year in the same institution. In the pilot study (21) urine samples without previous manipulation of 82 patients and 102 controls were analyzed by ELISA. EN2 was detected in 66% of PC patients and 12-15% of the controls and the ROC analysis showed an AUC of 0.80 for EN2 with sensitivity of 66% and specificity of nearly 90% using a cut-off of 42.5ng/ml. The second study published in 2012 (22) included the analysis of urine samples of 125 patients with clinically localized PC without any previous prostatic manipulation. An elevated level of EN2 was detected in 65–70% of the cohort, especially at more advanced tumor stages and there was a significant relation to tumor volume. No correlation to remaining patient and tumor characteristics was found. These results were not confirmed by Marszall et al (23) in 2015, who designed a study with 33 PC patients and 38 controls with benign prostatic hyperplasia confirmed by prostate biopsy. In this study urine samples were collected before and after prostatic massage and analyzed by ELISA. High EN2 levels were detected in 55% of PC patients before prostatic massage and 91% of patients after prostatic massage and in 47% of controls. The difference between both groups was only significant after prostatic massage with AUC=0.50 versus AUC=0.81. ROC analysis confirmed superiority over conventional serum PSA (AUC=0.77). A correlation to higher tumor stage and grade was found but only after prostatic massage samples. No healthy control group was included in this study. To test the influence of prostatic manipulation on the levels of EN2, we included a group of 40 patients enrolled for prostate biopsy which were later divided into biopsy-positive (n=20) and biopsy negative (n=20). No significant difference was found between the biopsy-positive and

biopsy negative groups and digital rectal examination did not increase urine EN2 levels. Normalization to urine creatinine did not change these results. Our work is the most comprehensive analysis on EN2 as a PC marker published till now. We designed a study including multi-brand assays, sample processing and examination by a single experienced operator in a blind manner, a cohort consisting of a control group, prostate cancer patients and biopsy-negative patients, multiple measurements of samples before and after prostatic manipulation normalized to urine creatinine. Among the limitations of our study are a relatively small cohort and the use of retrospective archived samples. Although it is a negative result, we believe it contributes to the current knowledge of this marker. In fact, the urinary EN2 test was licensed to Zeus Scientific as reported in 2013 with projections of submission to U.S. Food and Drug Administration in 1 year and worldwide clinical use in 2 years time (24). In 2018 an announcement by the developers of urinary EN2 was made of a new clinical trial set to have results available by 2019. So far no results were published and the trial was not yet registered at ClinicalTrials.gov (25).

We also decided to focus our attention on the early follow up of patients with adverse pathological features after RP. This patient group considered at high risk for BCR is very heterogenous with uncertain clinical courses. Overtreatment with ART is a reality that can affect 35-60% of these high-risk patients (26) besides possible adverse events including genitourinary and gastrointestinal toxicity. Additionally, the optimal timing of PSA testing after surgery is unknown and is of special importance in this group. Having this problematic in mind, we designed a study including 205 men harboring adverse pathological features after surgery with ultrasensitive PSA tests carried out at days 14, 30, 60, 90 and at 3-month intervals afterwards and the median follow up was 46 months. According to the final stratification model (AUC=0.76) patients with a PSA >0.068 ng/ml on day 30 or a PSA >0.015

ng/ml at day 60 would be indicated for early ART and the remaining would continue routine follow up. This would lead to a decrease in overtreatment from the initial 51% to 14%. The results obtained are in line with previous studies by Audenet et al (27) and Shen et al (28) that highlight the importance of early and intensive PSA analysis after RP to identify surgical failure. Also, ultrasensitive PSA assays offer a more precise measure of PSA and some studies report better BCR risk stratification than less sensitive assays (28). Eisenberg et al (29) showed that men with undetectable ultrasensitive PSA<0.01 ng/ml have a low risk of recurrence when compared to men with an undetectable conventional less sensitive PSA. No previous work examined the impact of the time between surgery and multiple early PSA levels on decision making and the current guidelines on ART are purely based on tumor characteristics following an 'adjuvant-radiotherapy to all high-risk' approach and excluding early PSA samples. Our study has some drawbacks including its modest follow up and cohort size, its retrospective design and lack of adjuvant radiotherapy-treated arm. Prospective studies with ultrasensitive PSA will further determine which patients will benefit from adjuvant therapy and which patients can be spared.

With an interest in improving the prediction and assessment of BCR pre- and postoperatively we designed a third study including 128 patients who underwent RP. Blood samples were collected before surgery, at 1 and 3 months post-RP. PSA, fPSA, %fPSA, [–2]proPSA, PHI and hk2 were the markers selected for analysis. The preoperative predictors of recurrence were PSA (AUC 0.64; p-value 0.029), [–2]proPSA (AUC 0.70; p-value 0.002) and most importantly PHI (AUC 0.73; p-value 0.0003). This finding is in line with previous works by Lughezzani et al. (30) and Maxeiner et al. (31) who tested preoperative PHI in cohorts of 313 and 437 patients respectively and confirmed its value as an independent predictor of BCR. In the postoperative period, PSA was the only marker that correlated with BCR at 1 month (AUC

0.69; p-value 0.0047) and 3 months (AUC 0.72; p-value 0.0004). This finding was in agreement with the work of S. De Luca et al. (32) who studied [–2]proPSA in a group of high-risk patients at 3-month intervals in the first year after RP to find it devoid of value. Contrarily, Casale et al (33) concluded that [–2]proPSA could be of use in detecting BCR earlier than PSA in a study with 134 patients after RP and a follow-up of three years. A low rate of BCR and a high rate of [–2]proPSA false positive results were cited as main limitations of the study. As far as we know, our study is the first recent work to test the remaining PSA isoforms in the postoperative period and their relation to BCR. Regardless of the recent advances brought by PSA isoforms such as [–2]proPSA and PHI in PC diagnosis, their use in prediction of disease recurrence is still budding and the available preoperative nomograms such as the MSKCC (34) or CAPRA score (35) that guide surgery and decision making concerning pelvic lymph node dissection and nerve-sparing techniques still use preoperative PSA.

Our study is original in testing isoforms of PSA at both preoperative and postoperative periods, and it highlights the value of preoperative [–2]proPSA and especially PHI at predicting BCR. Samples from a homogenous population were evaluated by the same operator in the same laboratory. Among the limitations of our work are a relatively small sample size and low rate of BCR and disease persistence and the use of stored serum samples. Cross-validation of models including [–2]proPSA and especially PHI in preoperative nomograms, on a larger population, in a prospective and multicentre setting seems the next logical step.

6. Conclusion

- The marker EN2 is devoid of clinical value in the detection of PC in contrast to what was previously reported.

- The sampling of PSA as early as day 30 after RP strongly correlates to BCR and ultrasensitive assays lead to better patient stratification for ART.
- [–2]proPSA and PHI outperform serum PSA as predictors of BCR and disease persistence preoperatively. Their inclusion in preoperative nomograms is the next logical step.

7. Summary

Prostate cancer is one of the most common malignancies in men with incidence rates up to 83.4/100.000. Until 1980s prostate cancer was only detected at a late stage almost always presenting with symptoms related to metastatic bone involvement. Prostate specific antigen was firstly used for monitoring response of patients to treatment until early 1990s when it was demonstrated that it could be used in the first line screening for PC. PSA lacks specificity and among its limitations are fluctuations with age, prostate size, inflammation and infection, recent manipulation, ejaculation and some medicaments. So far and despite these limitations no marker has been able to replace PSA and only few are proved to add to its sensitivity in both diagnosis and prognosis. The main aims of this dissertation thesis were to improve current diagnosis and prognosis of prostate cancer by studying a relatively unknown non-invasive urinary marker advocated to have high detection sensitivity and specificity, to explore the early postoperative PSA fluctuations and their relation to the occurrence of biochemical recurrence in a population at high risk and to study the role of PSA isoforms in predicting disease recurrence and disease persistence before and after radical prostatectomy. The results show that urinary Engrailed-2 is devoid of value in prostate cancer diagnosis and its measurement is highly dependent on the assay used. We demonstrated that evaluation of prostate specific antigen as early as day 30 in

a population of men bearing adverse pathological features after radical prostatectomy (and thus considered high-risk) is a good predictor of biochemical recurrence, leading to a considerable decrease in overtreatment with adjuvant radiotherapy in this patient group. The latter study determined that the isoforms [–2]proPSA and PHI outperform serum PSA in pre-surgery prognosis of biochemical recurrence. There is no value in testing these isoforms in the postoperative period and PSA continues to be the marker of choice at this time. We advocate further studies on the use of [–2]proPSA and PHI in preoperative nomograms instead of PSA and the combination with early ultrasensitive prostate specific antigen assays in patients at high-risk for biochemical recurrence.

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