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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

Disertační práce

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Poděkování

Velice děkuji svému školiteli doc. MUDr. Štěpánovi Veselému Ph.D. za jeho podporu, vědecký vstup a vedení. Děkuji prof. MUDr. Marku Babjukovi, CSc. že mi umožnil věnovat se postgraduálnímu studiu. Jejich široké znalosti a bohaté zkušenosti mě povzbuzovaly po celou dobu mého akademického výzkumu. Děkuji Ústavu lékařské chemie a klinické biochemie ve Fakultní nemocnici Motol, v osobě Mgr. Hany Lukšanové PhD. a prof. MUDr. Richarda Průšy, CSc. za zájem a obětavost v našem výzkumu a spolupráci při analýze vzorků. Ze všeho nejvíce děkuji své rodině, své matce, tetě a prarodičům za to, že mě vždy bezpodmínečně podporovali po mnoho let mimo domov a nyní nablízku.

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. Naše analýzy nepotvrdily klinickou užitečnost EN2 v moči 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. Jiné výsledky naznačují, že sérové PHI a [–2]proPSA má schopnost lepší predikce BCR než konvenční sérové PSA a použití těchto nových markerů v klinických predikčních modelech a nomogramech by mohlo významně přispět k časné detekci recidivy onemocnění. Analýza isoformem PSA však pravděpodobně nemá žádný význam ve sledování pacientů po operační léčbě PC.

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 after treatment. 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 strict follow-up protocol of ultrasensitive PSA (UPSA) at close time intervals. The ability of particular measurements 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 did not show any 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. Another results imply that serum PHI and [–2]proPSA outperforms conventional serum PSA in the prediction of BCR and the use of these novel biomarkers in clinical prediction models and nomograms would be most likely of a great value. On the other hand, there is probably 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

LIST OF ABBREVIATIONS

ADT	Androgen deprivation therapy
APC	Adenomatous polyposis coli
ARs	Androgen receptors
ART	Adjuvant radiotherapy
AUC	Area under the curve
BCR	Biochemical recurrence
BRCA2	Breast cancer 2
CAPRA	Cancer of the prostate risk assessment
CRPC	Castration resistant prostate cancer
csPC	Clinically significant prostate cancer
DCEI	Dynamic contrast enhanced imaging
DHT	Dehydrotestosterone
DP	Disease persistence
DRE	Digital rectal examination
DWI	Diffusion weighted imaging
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EPE	Extraprostatic extension
fPSA	Free PSA
GSTP1	Glutathione S-Transferase Pi 1
Her 1 (ErbB-1)	Human Epidermal growth factor receptor 1 (ErbB-1)
Her 2/neu (ErbB-2)	Human Epidermal growth factor receptor 2/neu (ErbB-2)
Her 3 (ErbB-3)	Human Epidermal growth factor receptor 3 (ErbB-3)
Her 4 (ErbB-4)	Human Epidermal growth factor receptor 4 (ErbB-4)
hK2	Human kallikrein 2
HOXB13	Homeobox B13
HSP	Heat shock protein
IGF	Insulin growth factor
ISUP	International Society of Urological Pathology
LNI	Lymph node invasion
MAPK	Mitogen-activated protein kinase
mpMRI	Multiparametric magnetic resonance

mRNA	Messenger RNA
MSKCC	Memorial Sloan Kettering cancer centre
mTOR	Mammalian target of rapamycin
NNS	Number needed to screen
PBCG-RC	Prostate Biopsy Collaborative Group – risk calculator
PC	Prostate cancer
PCSM	Prostate cancer specific mortality
PDK1	Phosphoinositide-dependent protein kinase 1
PI-RADS™v2	Prostate imaging reporting and data system 2015 version 2
PHI	Prostate health index
PIP2	Phosphatidylinositol-4, 5-bisphosphate
PIP3	Phosphatidylinositol-3, 4, 5-triphosphate
PI3K	Phosphoinositide 3 kinase
PSA	Prostate specific antigen
PSADT	Prostate specific antigen doubling time
PSM	Positive surgical margin
PSMA	Prostate-specific membrane antigen
PTEN	Phosphatase and tensin homolog
RASSF1	Ras Association Domain Family Member 1
ROC	Receiver operating characteristic
RP	Radical prostatectomy
RT	Radiotherapy
S3M	Stockholm-3 model
SRT	Salvage radiotherapy
SVI	Seminal vesical invasion
TNM	Tumor, node, metastasis
TP	Transperineal
TP53	Tumor protein 53
TR	Transrectal
TRUS	Transrectal ultrasound
US	Ultrasound
USFDA	United States Food and drug administration
USPSTF	United States Preventive Services Task Force

TGF-β	Transforming growth factor-beta
wbMRI	Whole body MRI

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1. INTRODUCTION

1.1. Epidemiology

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 people[1]. The lifetime risk of PC diagnosis was found to be 13.2–15.0% for Caucasians, 23.5–37.2% for Africans and 6.3–10.5% for Asians in a population study conducted in the United Kingdom[2]. The highest incidence rates occur in Northern and Western Europe, North America, Australia, and New Zealand, the Caribbean and South Africa and the highest mortality rates occur in populations of African descent (Caribbean and Sub-Saharan Africa) (Fig.1).

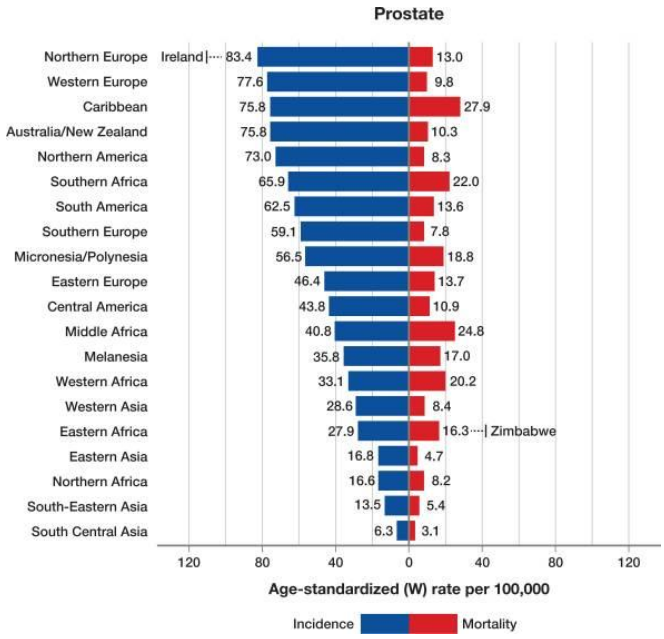

Fig 1**:** Region-specific incidence and mortality. Age-standardized rates for PC in 2020. Rates are shown in descending order of the world (W) age-standardized incidence rate, and the highest national age-standardized rates for incidence and mortality are superimposed. Figure and caption from [1]

As shown, the PC incidence rate varies widely between different geographical areas and besides biological, genetic and lifestyle factors, it is largely affected by PSA testing, screening programs, the aging population, and the number of procedures for benign prostatic hyperplasia with incidental PC diagnosis[3]. Screening has led to an increase in the incidence of PC without necessarily being associated to a decrease in cancer specific

mortality. There is thus no current international consensus on the recommendation of PSA testing and early diagnosis can lead to harm due to overdiagnosis and unnecessary treatment leading to reduced quality of life [4].

1.2. Etiopathogenesis

PC develops mainly from malignant changes of the epithelium and is classified as a carcinoma normally affecting the peripheral zone of the prostate. Rarer types of prostate cancer include sarcomas and lymphomas[5].The epithelium is composed of luminal, basal, and rarer neuroendocrine cells (Fig 2).

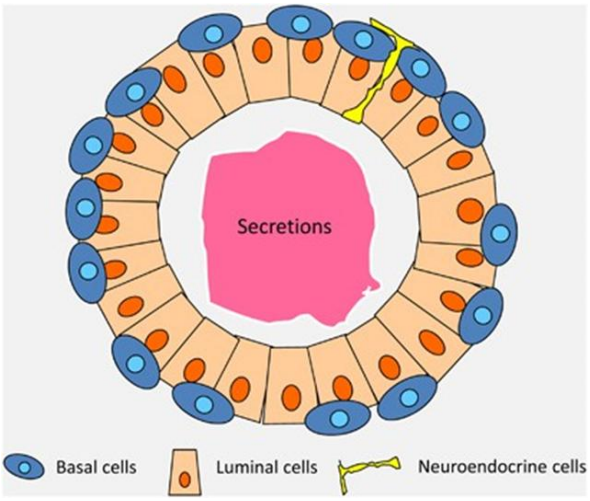

Fig. 2: Normal prostate epithelium. Figure from [6]

Luminal cells express androgen receptors (ARs) and secrete PSA, while basal and neuroendocrine cells are devoid of ARs and are thus androgen independent. PC growth and progression is on an initial phase dependent on AR activation via testosterone and dihydrotestosterone, inducing nuclear translocation of the AR and binding to androgen response elements initiating the transcription of genes necessary for cell proliferation (Fig.3). Peptide growth factors such as epidermal growth factor (EGF), transforming growth factor-β (TGF-β) and insulin-like growth factor (IGF) facilitate the AR-proliferation of epithelial cells. EGF family of growth factors interact with their receptors including ErB-1 or Her 1 (EGFR), Her 2/neu (ErbB-2), Her 3 (ErbB-3) and Her 4 (ErbB-4)[7]. For instance, the expression of EGFR is low in normal prostate tissue and elevated in primary PC tissue and metastatic PC tissues [8].

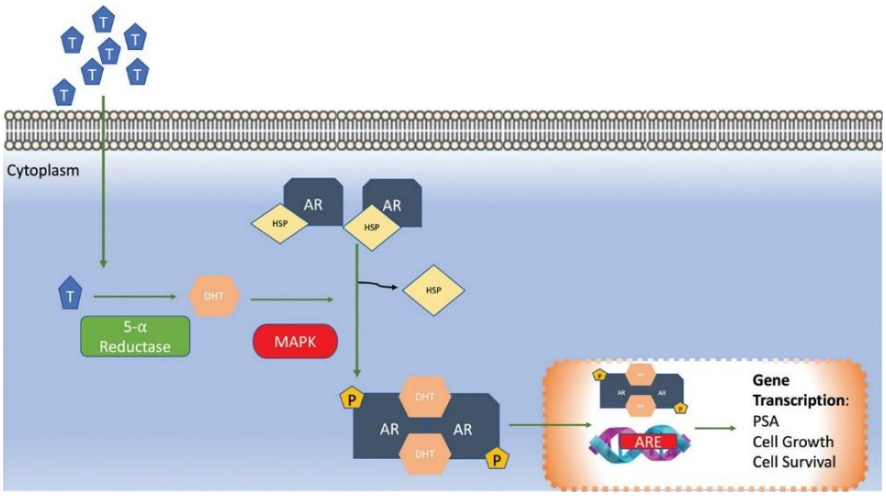

Fig. 3: Androgenic regulation of PC. In the cytoplasm, activity of ARs is regulated by ligand-binding and heat shock proteins (HSP). Testosterone (T) is transported into the cytoplasm of androgen-receptive cells and is converted to DHT by 5-α reductase. DHT ligand binding leads to dissociation from HSP, MAPK then phosphorylates the receptor, this is followed by dimerization. The AR dimer then translocates into the nucleus where it binds to androgen response elements (AREs) in the DNA activating transcription of elements that are essential for cell growth and survival. Figure and caption from [9]

Elevated levels of EGF and EGFR are produced by prostate cancer cells but also by stromal cells/fibroblasts, leading to growth and survival of PC cells even in the absence of androgens. Overexpression of Her 2 kinase increases the AR expression and promotes growth of prostate cancer cells resistant to hormonal castration (castration resistant PC) [10] (Fig 4).

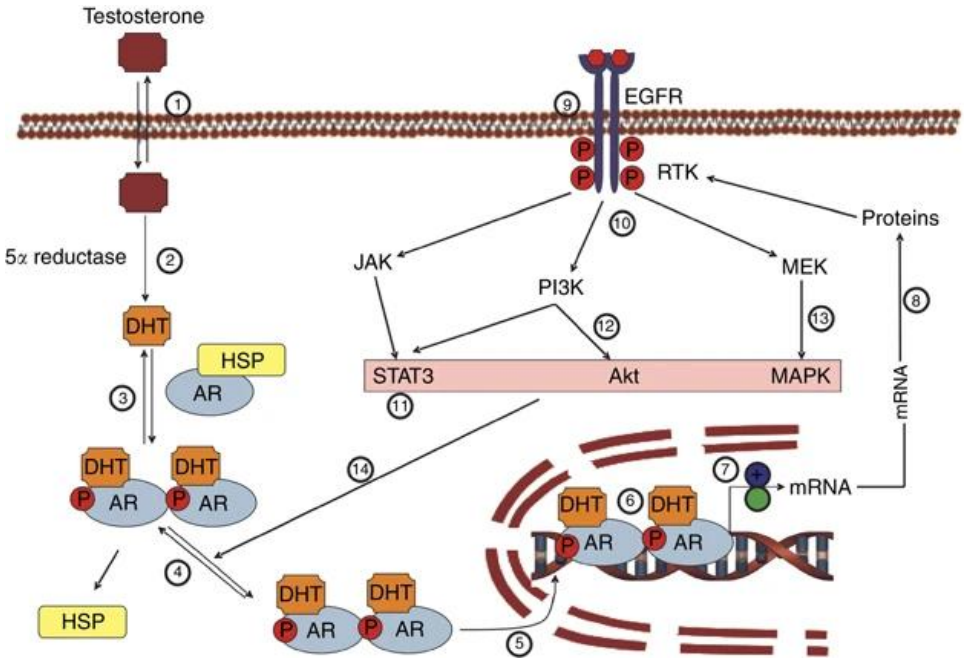

Fig. 4**:** Androgen-receptor signaling in normal prostate and prostate cancer and proposed interaction with EGFR. Reaction 1: unbound testosterone crosses the plasma membrane by diffusion. Reaction 2: Inside the prostate cell testosterone is converted to 5α-dihydrotestosterone (more potent) by 5α-reductase. Reactions 3 and 4: 5α-DHT binds to the AR and causes its activation and transformation that includes dissociation from heat shock proteins, dimerization and phosphorylation. Reaction 5: The complex 5α-DHT-AR translocates into the nucleus and interacts with the androgen response element (reaction 6). This leads to recruitment of coactivators or corepressors to regulate gene expression (reaction 7). It's postulated that in the normal prostate, the activation of AR results in downregulation of EGFR mRNA (reaction 7) resulting in reduced EGFR protein synthesis (reaction 8) and reduction of the functional protein (reaction 9). In PC it is proposed that a molecular switch regulating gene expression is turned off resulting in increased mRNA and EGFR protein synthesis. Further in PC, activation of EGFR by EGF results in signaling leading to activation of AR even in the absence of 5α-DHT (reactions 10-13). This results in tumor androgen independence. Figure and caption from [11]

Further, the loss of tumor suppressor genes such as TP53 and PTEN impair the normal AR regulation, increase cellular proliferation, and lead to reduced apoptosis (Fig 5).

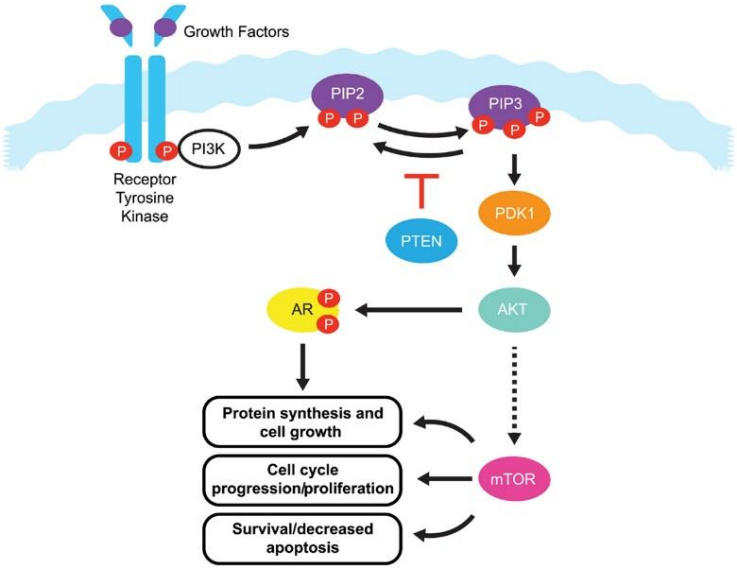

Fig. 5: Role of PTEN gene in prostate cancer growth. Growth factors bind to receptor tyrosine kinases that recruit and activate phosphoinositide 3-kinase (PI3K). Activated PI3K converts phosphatidylinositol-4, 5-bisphosphate (PIP2) to phosphatidylinositol-3, 4, 5-triphosphate (PIP3) which phosphorylates Akt via phosphoinositide-dependent protein kinase 1 (PDK1). One of Akt's most important targets is mTOR involved in cell growth, proliferation and survival. Activated Akt also interacts with the AR in an androgen independent manner leading to overactivation of the AR pathway in castration resistant prostate cancer (CRPC). PTEN is a tumor suppressor that negatively regulates the pathway by removing a phosphate group from PIP3, converting it to PIP2. Therefore, loss of PTEN leads to overactivation of Akt and uncontrolled cell growth, decreased apoptosis and enhanced tumor angiogenesis. Figure and caption taken from [12]

Only three risk factors for PC are defined: age, race and positive family history. Family history and ethnicity are associated with a genetic predisposition to prostate cancer. True hereditary prostate cancer (> 3 cases in the family, presence in 3 successive generations, or > 2 men diagnosed with PC < 55 years) is estimated to represent 5–15% of all cases[13] and is associated with an earlier onset (6-7 years) with similar clinical course and aggressiveness. Men of African descent have higher incidence of PC, are diagnosed with more advanced disease, and have worse outcomes. The most relevant germline mutations associated with PC involve the following genes: BRCA1, BRCA2, ATM, HOB13, PALB2, CHEK2, mismatch repair genes[14]. Although many studies exist on the effects of diet[15],obesity [16], metabolic syndrome [17]hypercholesterolemia[18] , some medications (Metformin [19] and 5-alpha-reductase inhibitors [20]), occupational risk factors[21] and ejaculation frequency[22] no specific measures are recommended to decrease the risk of PC.

1.3.Grading and staging

Diagnosis of PC is made by prostate biopsy and histologically there is loss of basal cells, loss of gland architecture, disruption of epithelial-stromal basement membrane and nuclear atypia[23]. Aggressiveness of the disease is graded according to the Gleason score system[24] based on the degree of differentiation of the tumor cells (fig. 6). In the original grading system, 5 grades were described (1-5) but in the more recent modifications by the International Society of Urological Pathology (ISUP) grade 1 and grade 2 were eliminated[25, 26]. Currently, Gleason score is calculated by adding the most extensive primary grade to the second most common Gleason pattern or by duplication in case only one pattern is present. In case three patterns are present, the score includes the most common grade followed by the highest grade, regardless of its extent. In 2014 the ISUP proposed its own grading system stratifying histological findings according to prognostic behavior [26] (Tab. 1). The staging of prostate cancer is performed according to the 2018 Clinical Tumour Node Metastasis (TNM) classification [27] (Tab. 2).

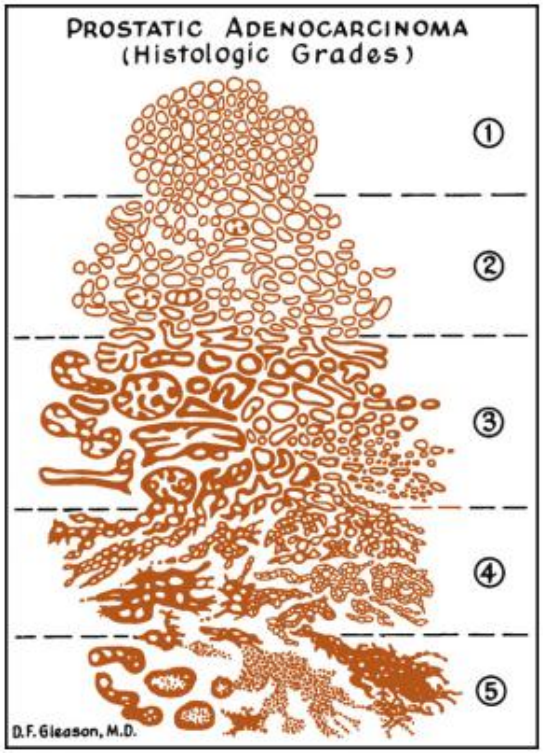

Fig. 6: Prostate cancer grading. Figure taken from [28]

Table 1: International Society of Urological Pathology 2014 grade (group) system

Gleason score	ISUP grade
2-6	1
7 (3+4)	2
7 (4+3)	3
8 (4+4 or 3+5 or 5+3)	4
9-10	5

Table. 2: Clinical Tumor Node Metastasis (TNM) 2018 classification of PC [27]

T - Primary Tumour (stage based on digital rectal examination [DRE] only)		
TX	Primary tumour cannot be assessed	
T0	No evidence of primary tumour	
T1	Clinically inapparent tumour that is not palpable	
	T1a	Tumour incidental histological finding in 5% or less of tissue resected
	T1b	Tumour incidental histological finding in more than 5% of tissue resected
	T1c	Tumour identified by needle biopsy (e.g. because of elevated prostate-specific antigen [PSA])
T2	Tumour that is palpable and confined within the prostate	
	T2a	Tumour involves one half of one lobe or less
	T2b	Tumour involves more than half of one lobe, but not both lobes
	T2c	Tumour involves both lobes
T3	Tumour extends through the prostatic capsule	
	T3a	Extracapsular extension (unilateral or bilateral)
	T3b	Tumour invades seminal vesicle(s)
T4	Tumour is fixed or invades adjacent structures other than seminal vesicles: external sphincter, rectum, levator muscles, and/or pelvic wall	
N - Regional (pelvic) Lymph Nodes[1]		
NX	Regional lymph nodes cannot be assessed	
N0	No regional lymph node metastasis	
N1	Regional lymph node metastasis	
M - Distant Metastasis[2]		
M0	No distant metastasis	
M1	Distant metastasis	
	M1a	Non-regional lymph node(s)
	M1b	Bone(s)
	M1c	Other site(s)

1.4. Current diagnosis

The decision to mass screen for PC has been controversial leading to recommendation changes over the years. An initial aggressive widespread screening approach using PSA led to an increase in false positive results, psychological distress, additional tests and prostate biopsies with its main associated risks (bleeding and infection), substantial overdiagnosis of indolent cases and disease overtreatment. In 2012 the United States Preventive Services Task Force (USPSTF) recommended against PSA screening[29]. The decrease in the use of PSA was associated with an increase in the number of patients with advanced disease and metastatic disease at diagnosis [3] similar to the pre-PSA era. Some recent data suggest a benefit of PSA testing in reducing the cancer specific mortality[30] and the longer the follow up (16 years) the larger the absolute benefit [31]. Repeated screening may thus reduce PC mortality on a population level.

Current diagnosis of PC is still performed by PSA and digital rectal examination. There are additional (more recent) roles of imaging methods, PSA derivatives and other biomarkers (in serum, urine and tissue).

The present recommendation [32] is to offer PSA testing to well informed men with a life expectancy of 10-15 years. Candidates for screening are men 50 years or older, or 45 years from African descent or with a positive family history for PC. Carriers of BRCA2 mutations should be tested from the age of 40 years. Alternatively, an individualized strategy can also be adopted based on the baseline PSA level at 40 years and at 60 years. In this case, individuals with a PSA level >1 ng/ml at 40 years and >2 ng/ml at 60 years are considered at higher risk, and PSA testing should be performed every two years. Otherwise follow up should be in 8 years' time for those considered low risk.

PSA is a continuous parameter and the higher the level, the higher the suspicion of prostate cancer (Table. 3) [33]. A level of PSA comprehended between the values 3-10 ng/ml should prompt a repeat analysis 4-7 weeks later [34]. For a cut-off of 4ng/ml PSA has sensitivity of 67.5-80% [35, 36] and specificity of 60-70% [36] PSA has several diagnostic limitations that have led to the research of new markers and imaging methods able to improve PC diagnosis and prognosis. PSA is organ specific but not prostate cancer specific, it fluctuates with age, prostate gland size, inflammation, infection, recent manipulation or biopsy, ejaculation and vigorous exercise prior to testing and some medicaments.

Table. 3 Risk of PC identified by systemic PC biopsy in relation to low PSA

PSA level (ng/mL)	Risk of PC (%)	Risk of ISUP grade >2 PC (%)
0,0-0,5	6,6	0,8
0,6-1,0	10,1	1,0
1,1-2,0	17,0	2,0
2,1-3,0	23,9	4,6
3,1-4,0	26,9	6,7

Digital rectal examination alone can diagnose approximately 18% of PC cases[37] and it is the only approved tool for clinical staging.

PSA derivatives such as density (ng/ml/cc) and the ratio free/total PSA (%fPSA) can help predict the presence of PC in addition to total PSA[38, 39]. Other PSA derivatives such as velocity (ng/ml/year) and PSA doubling time have a role in prognosis during treatment.

Prostate health index (PHI) consists of the formula [–2]proPSA/fPSA∗ √PSA in which [–2]proPSA is a precursor of PSA. PHI is a decision tool used at both initial and repeat biopsy settings. It predicts significant PC with accuracy, and it outperforms the specificity of both PSA and %fPSA[40, 41] [42].

The current recommendation for prostate biopsy in men with a normal DRE and a PSA between 3–10 ng/mL is to add an additional exam, a reflex test, such as a risk calculator, a magnetic resonance or a serum, urine or tissue biomarker test.

1.4.1. Currently approved serum markers and prediction models

Both the 4Kscore Test and PHI are used at both decision for initial biopsy (biopsy-naïve patients) and at repeat biopsy settings.

The 4Kscore Test consists of four parameters including hk2 and hk3 (the dominant kallikreins expressed in prostate tissue), intact PSA and free PSA and relevant clinical information (age, DRE and previously negative biopsy). In PC there is dysregulation and overexpression of hK2 and hK3 and in undifferentiated cancers of intact PSA and hK2 [43]. These kallikreins alter the regulation of cell growth, cell invasion and angiogenesis

which characterizes aggressive prostate cancer and are thus good markers of PC progression and metastases risk[44]. Therefore, it can help identify men with aggressive PC at risk of metastases and death from PC versus those with indolent forms of cancer that can avoid biopsy and/or active treatment (interventions) The 4Kscore Test correlates to both biopsy and radical prostatectomy samples[45, 46] and it is the only test except serum PSA that correlates to the long-term endpoint PC metastases[47]. IsoPSA is an approved test that detects structural isoforms of PSA with an aqueous 2- phase system. It outperforms total and %fPSA in accuracy, specificity and predictive value at detecting PC and high-grade PC at biopsy with similar sensitivity[48].

The Stockholm-3 model (S3M) is a prediction model that combines clinical information (age, previous biopsy, family history of PC) with blood (PSA, fPSA, %free PSA, hK2, macrophage inhibitory cytokine-1, and microseminoprotein) and genetic markers (254 single-nucleotide polymorphisms and an explicit variable for HOXB13) and prostate examination (DRE and prostate volume). It demonstrates a 32% reduction in prostate biopsies [49]

The Rotterdam PC risk calculator is another validated tool used in biopsy-naïve and repeat-biopsy settings based on various patient characteristics such as PSA, prostate volume and previous biopsy status. It improves the detection of any PC, indolent PC and csPC (ISUP≥2 with invasive cribriform and/or intraductal carcinoma) leading to a 20% decrease in the number of biopsies at the expense of a 2% failure to diagnose csPC (2%) [50] .

The Prostate Biopsy Collaborative Group risk calculator (PBCG-RC) is another validated risk tool based on routine clinical characteristics (race, age, PSA, DRE, prior biopsy and family history) [51]

1.4.2. Currently approved urine markers and prediction models

Prostate cancer gene 3 (PCA3) is located on chromosome 9 and it's transcribed to non-coding mRNA which is overexpressed 60-100 times in prostate tumour cells when compared to normal prostate cells [52]. The PCA3 score (commercially available as Progensa^{TM} test) is calculated after three strokes of prostatic massage and is a ratio between PCA3 mRNA and PSA mRNA both detected in urine (PCA3 mRNA/PSA mRNA × 1000).

Its current clinical indication is to help decide if a repeat biopsy is needed in men with elevated PSA and a previous negative biopsy [53] outperforming in this setting PSA and %fPSA with superior AUCs. The use of the assay in the primary setting is still a subject of study [54].

SelectMDX is a urine molecular test that combines the expression of HOXC6 and DLX1 mRNA with clinical risk factors to detect PC and high-risk PC[55]. It demonstrates high sensitivity (90% for high-grade cancers) in the primary setting and helps select men for further investigations (mpMRI and/or biopsy). A recent study comparing SelectMDX biopsy strategy versus a mpMRI biopsy strategy was in favour of the mpMRI strategy with a higher number of biopsies avoided and a higher number of high-grade cancers detected [56].

Other urine-based tests and model scores (ExoDx Prostate IntelliScore and Michigan prostate score) are still investigational [57, 58].

1.4.3. Currently approved tissue markers

There are currently no approved tissue biomarkers in the diagnostic setting. The available data refer to ConfirmMDX that can detect epigenetic methylation changes in three genes of benign prostatic tissue (Methylated APC, RASSF1 and GSTP1) indicating the presence of carcinoma [59].

1.4.4. Current diagnostic imaging methods

1.4.4.1. TRUS

Transrectal ultrasound (TRUS) is not a reliable method to detect PC [60]. On TRUS 60–70% of PC lesions are hypoechoic, 30-40% are isoechoic and a small percentage are hyperechoic [61]. Hypoechoic lesions are not PC specific as hyperplasia, HGPIN, prostatitis and necrosis can have the same appearance. There is no proven benefit of additional biopsies of these areas [62] besides the regular biopsy scheme. Advanced ultrasound (US) techniques such as the use of Doppler signal, contrast-enhanced US, elastography, microbubble contrast agents are under study [63] but lack large scale validation and are reader-dependent.

1.4.4.2.MRI

Multiparametric MRI (mpMRI) has received great attention in recent years and has changed the guidelines for diagnosis of PC. Besides regular MRI, it combines T2 images with diffusion weighted images (DWI) and dynamic contrast enhanced images (DCEI) to detect PC more accurately. It is classified with a five-risk category score system PI-RADS™v2 [64] (**Erro! A origem da referência não foi encontrada.**). MRI is considered negative in case PIRADS<3 and positive in case of detecting a lesion PIRADS≥3. It is especially sensitive to identify ISUP>2 cancers larger than 10mm [65, 66] and less sensitive for ISUP 1 PC identifying less than 30% of these sized <0.5cc [67].

Table 4: PI-RADS ™v2 assessment categories

	Risk category
PIRADS 1	Very low (clinically significant PC is highly unlikely to be present)
PIRADS 2	Low (clinically significant PC is unlikely to be present)
PIRADS 3	Intermediate (the presence of clinically PC disease is equivocal)
PIRADS 4	High (clinically significant PC is likely to be present)
PIRADS 5	Very high (clinically significant PC is highly likely to be present)

The current recommendation [32] is to perform a mpMRI in all patients (both biopsy-naïve and with previous negative biopsy) before performing a prostate biopsy. In case of unavailability, a systematic biopsy scheme is indicated. All biopsy-naïve patients with lesions classified as PIRADS ≥3 should undergo both a systematic and target-lesion biopsy. In case the PIRADS classification is <3 and the clinical suspicion for PC is low, the prostate biopsy can be withheld based on a shared decision with the patient. All repeat-biopsy patients with lesions PIRADS ≥3 can undergo a target-lesion only biopsy and in case the MRI is negative (PIRADS<3) and the clinical suspicion is high a systematic biopsy should be considered with the patient.

Despite its revolutionary role in PC diagnostics, issues such as cost, widespread availability and inter-reader variability remain.

1.4.4.3.Prostate biopsy

The current biopsy recommendation [32] is to perform a minimum of 8-cores in a 30cc sized prostate and 10-12 cores in larger sized prostates (**Erro! A origem da referência não foi encontrada.**) focusing on far lateral areas. Increasing the total core number to >12 cores has not showed a significant improvement in PC diagnosis [68–70].

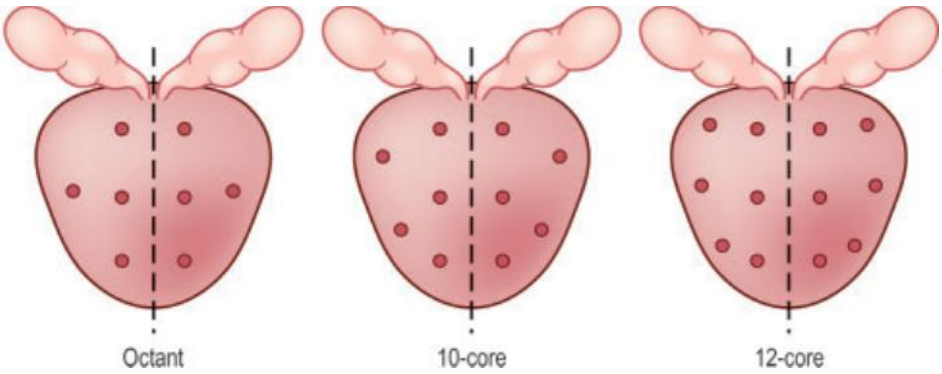

Fig. 7: Prostate biopsy schemes. Figure taken from [61]

Additional cores should be taken from suspicious areas identified on DRE or mpMRI (by cognitive, direct in bore or ultrasound software-fused imaging). The biopsy can be performed with a transrectal (TR) probe or it can be performed via the perineal route (TP). The TP route has less infectious complications, no rectal bleeding and increased detection rate of csPC (86% versus 73% of the TR route) [71] especially in tumours located in the anterior part of the prostate (Fig. 8, Fig. 9).

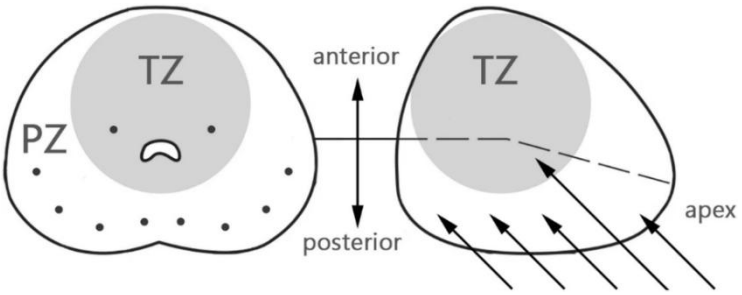

Fig. 8: Transrectal prostate biopsy mapping. PZ – peripheral zone, TZ – transitional zone.Figure taken from [72]

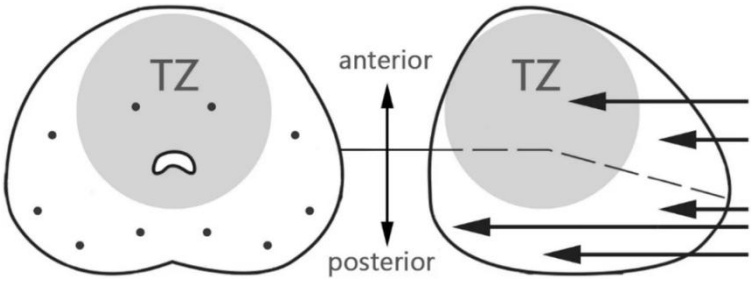

Fig . 9: Transperineal prostate biopsy mapping. PZ – peripheral zone, TZ – transitional zone. Figure taken from [72]

1.5.Prostate cancer staging

In all patients clinical T staging is assessed by DRE and imaging findings are not yet considered. Imaging methods are used to determine the local extent of the disease, the presence lymph node involvement and distant metastases and guide management.

A pre-biopsy MRI should be performed in all patients and T2 weighted imaging can give important information regarding seminal vesical invasion (SVI) and extraprostatic extension (EPE).

Low risk disease (PSA<10ng/ml, ISUP1 and cT1-cT2a) does not require any additional staging methods.

Intermediate risk disease (PSA 10-20 ng/ml or ISUP2/3 or cT2b) with ISUP3 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 .[32].

PSMA PET/CT is a new molecular imaging method where the radioactive tracer 68Ga-PSMA-11 is injected and attached to PSMA proteins (overexpressed in PC cells). PSMA PET/CT is a new molecular imaging method where the radioactive tracer 68Ga-PSMA-11 is injected and attached to PSMA proteins (overexpressed in PC cells). PSMA PET/CT has a high per-patient sensitivity and specificity, and it is the most accurate N-staging and M1b-staging method outperforming CT, MRI, wbMRI and choline PET/CT [73, 74]. Since RCTs including these new imaging methods such as Choline PET/CT, PSMA PET/CT and wbMRI are ongoing, there is still no outcome data of the treatment changes their use would bring and all patients are currently managed based on conventional imaging methods (CT/MRI and bone scan).

1.6. Preoperative nomograms

Accurate prediction of pathological stage including organ-confined disease, seminal vesicle involvement, extraprostatic extension, lymph node invasion is of upmost importance to plan treatment of PC patients. Studies were made by grouping patients with similar preoperative characteristics to predict disease behavior.

Partin tables [75] were originally published in 1993 using data from patients treated between 1982 and 1991 at John Hopkins, most when PSA was not available. Recent updates of the Partin tables [76–78] reflect the 'stage migration' of PC from systemic and locoregionally advanced at diagnosis to organ confined disease either by earlier detection or changes in disease biology. The 'Partin tables' predict the disease pathological stage at radical prostatectomy according to commonly available preoperative data (clinical stage, PSA and biopsy Gleason score). After Partin tables other nomograms have been developed (Fig. 10) .[79]

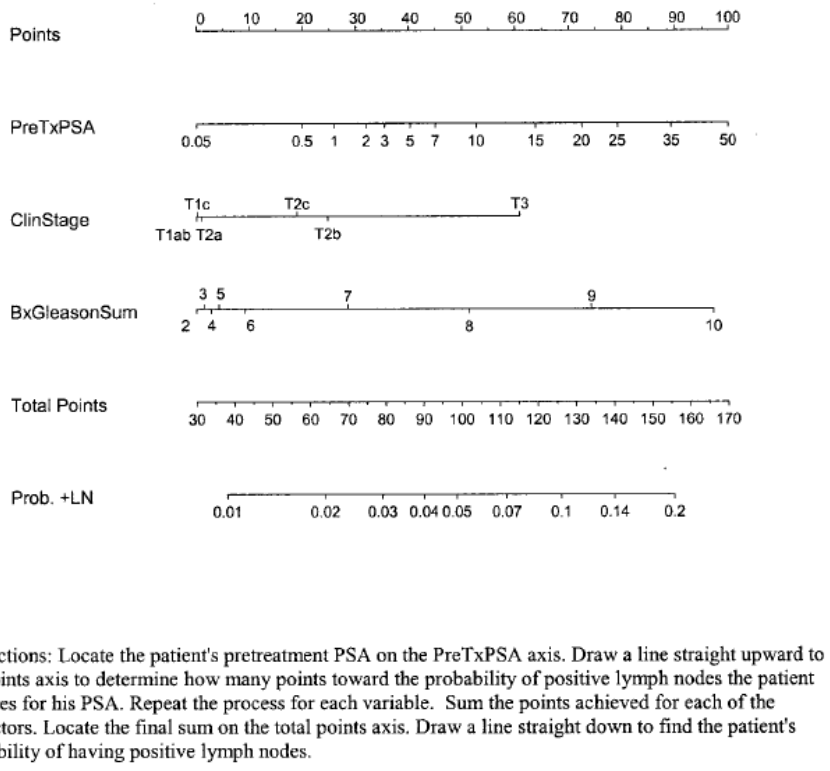

Fig. 10: Variable nomogram to predict probability of positive lymph nodes according to Cagiannos et al [79]

The MSKCC nomogram [79] predicts the risk of lymph node metastases and can be used to avoid lymphadenectomy in low-risk patients decreasing morbidity and treatment cost using the same preoperative parameters.

The Briganti nomogram [80] (Fig.11) considers the previous parameters (PSA, clinical stage and biopsy Gleason score) and adds the percentage of positive biopsy cores as a covariate.

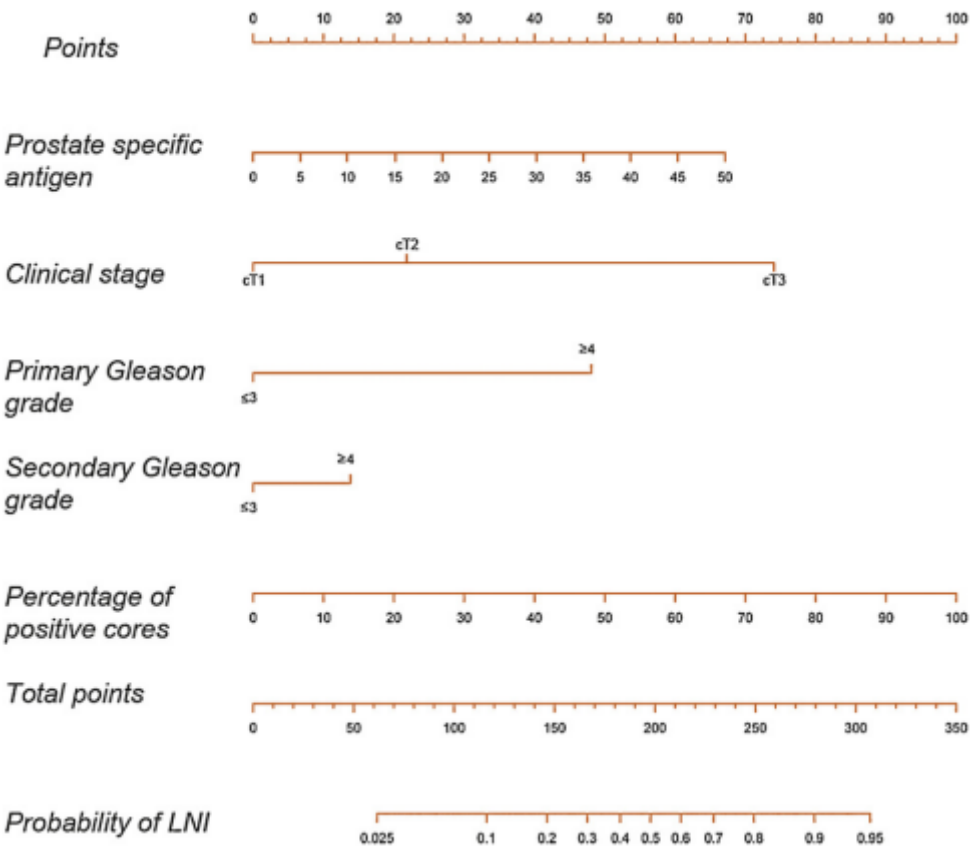

Fig. 11: Briganti nomogram. Nomogram that predicts the probability of lymph nodes invasion (LNI) in patients undergoing extended pelvic lymphadenectomy based on pretreatment parameters. [80]

All previous nomograms have similar capabilities and accuracy [81] and are approved [32]for predicting lymph node invasion (LNI) and performing pelvic lymph node dissection in positive cases with a cut-off of 5%.

An updated nomogram[82, 83] (Fig. 12) has been recently approved including the findings of pre-biopsy MRI. Based on this nomogram patients can be spared of pelvic lymph node dissection if the risk of lymph node involvement is <7%.

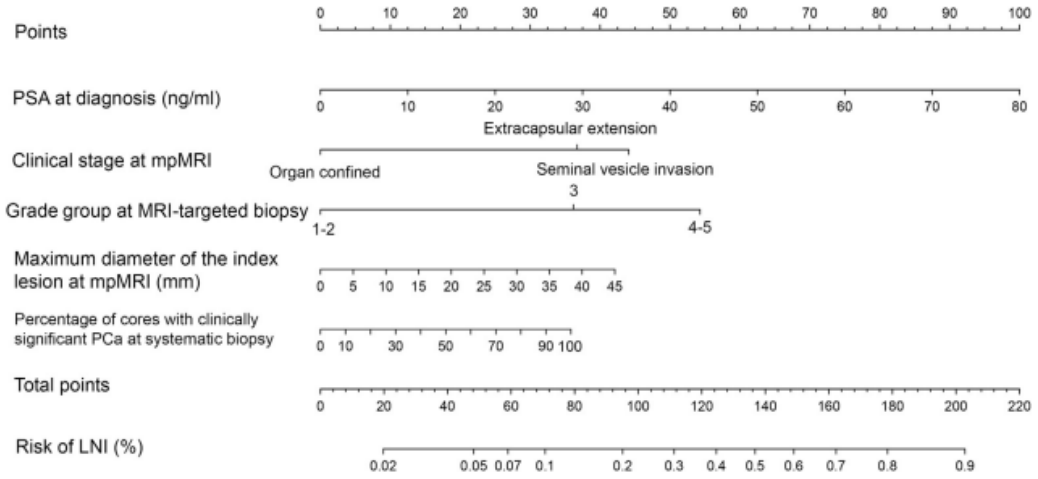

Fig. 12: Novel nomogram predicting the probability of lymph node invasion (LNI) for patients diagnosed via targeted biopsies and treated with radical prostatectomy and extended pelvic lymph node dissection according to Gandaglia et al[83]

1.7.Overdiagnosis

Evidence of over detection of PC is the ratio of annual incidence to disease specific mortality. In 2020 the global incidence for PC was 30.7/100.000 males and the mortality was 7.7/100.000 males while the western Europe incidence [84] for PC was 77.6/100.000 males and the mortality rate 9.8/100.000 males. Also, the lifetime risk of PC (11.6% i.e. 1 in 9 men) is very different from the lifetime risk of PC death (2.4% i.e. 1 in 41 men) [85]. It is of note that the lifetime risk of PC would be even higher if all men underwent screening on a regular basis.

1.8. Current management

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 localized disease can be managed with active surveillance or active treatment: radical prostatectomy (open, laparoscopic or robotic), radiotherapy or low dose-brachytherapy.
Active surveillance consists of regular follow up with programmed PSA samples, digital rectal examinations, MRI imaging and repeat biopsies. This is indicated for patients with low-risk disease who wish to avoid unnecessary treatment with potential side effects and wish to be treated at the correct timing without compromising survival [86].

Intermediate-risk PC patients with localized disease can be managed by radical prostatectomy (open, laparoscopic or robotic) with or without pelvic lymph node dissection (in case the risk of LNI is below the threshold in the nomogram), radiotherapy accompanied by 4-6 months of treatment with androgen depriving therapy (ADT) or a combination of brachytherapy and radiotherapy with 4-6 months of ADT. Highly selected patients with intermediate-risk disease ISUP 2 can be offered active surveillance (< 10% Gleason pattern 4, PSA <10 ng/mL, < cT2a, low disease extent on imaging and low biopsy extent) or low-dose brachytherapy[32].

High-risk localized PC patients can be managed with surgery (open/laparoscopic or robotic radical prostatectomy with pelvic lymph node dissection), radiotherapy plus 2-3 years (long term) ADT or a combination of radiotherapy with a brachytherapy boost plus long-term ADT.

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

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 [32] In cases low-volume M1 disease, prostate radiotherapy in addition to ADT can be beneficial [87].

1.8.1. Adjuvant and salvage treatments

After the primary form of therapy patients are surveilled with PSA.

After radical prostatectomy the level of PSA should be undetectable at 4-8 weeks post-surgery [88]. Patients should have measurements of PSA every 6 months for 3 years and then yearly[89] .

After radiotherapy, the PSA level decreases more slowly than post-RP. It can take 3 years or more to achieve a nadir level. The National Comprehensive Cancer Network advises testing every 6 to 12 months for 5 years and then annually[90]

Adjuvant treatments are defined as additional to the initial therapy with the objective of reducing the risk of recurrence. The current indications for adjuvant treatment [32] 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 (pN) 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.

Salvage treatments are applied when biochemical recurrence (BCR) occurs.

1.8.1.1.After primary surgery

For BCR post-radical prostatectomy salvage radiotherapy (SRT) is the recommended option. It should be initiated in patients with a rapid PSA kinetics after RP and with a PSA cut-off of 0.4 ng/mL [91](6). The optimal dose is not defined but a minimum of 64 Gy to the prostatic fossa and base of seminal vesicles (depending on the pathological stage) in combination with ADT is recommended [32] In high-risk patients, whole pelvis irradiation is recommended opposed to prostatic fossa SRT-only [92].

Salvage ADT (for the duration of ≥2 years) after SRT for BCR for high-risk patients has shown better MFS and DSS versus observation for patients with PSADT <6months [93]. The rationale for the use of SRT versus adjuvant radiotherapy is that waiting for PSA relapse is unlikely to compromise oncological outcomes, some patients can be spared of radiotherapy, and potential treatment side effects will be delayed.

1.8.1.2.After primary radiotherapy

Therapeutic options in these patients are monitoring, salvage local procedures or ADT (continuous or intermittent).

PSA monitoring can be an option in patients at low risk of BCR, unfit patients with a short life expectancy <10 years or patients unwilling to undergo salvage treatments.

Salvage RP should only be considered in selected patients and in specialized centers since the rate of adverse events is high compared to primary surgery [94]. Other salvage local treatments include brachytherapy, cryoablation and high-intensity focused ultrasound. The data on these is still poor and they should only be offered in experienced centers.

It is yet unclear the optimal timing of salvage ADT and whether it should be administered in a continuous or intermittent form. Current recommendations [32] do not advise it for M0 patients with a PSADT>12months.

1.8.2. Treatment side effects

Most patients after radical prostatectomy are affected by erectile dysfunction [95] with rates of insufficient erections for intercourse at 2 and at 5 years post-RP 78-87% [96]. Risk factors for postoperative ED include non-nerve sparing surgery, surgeon's experience, age of the patient, baseline sexual function, smoking, diabetes, and hypertension [97]. Although the concept of penile rehabilitation has recently gained importance, clinical studies report conflicting results and the optimal program is still unknown [98].

Urinary incontinence affects around 20% of men 12 months after radical prostatectomy [99] and about 8.4% men continue to have severe incontinence within 18 months of the surgery [100].

Laparoscopic and robotic RP have failed to show a superiority over open RP in short and long term oncological and functional outcomes [101, 102].

Late adverse events after radiotherapy include mainly genitourinary and gastrointestinal toxicity including proctitis, rectal bleeding, increased stool frequency, increased daytime and night-time urinary frequency hematuria, urinary incontinence, dysuria and acute urinary retention [103]. Sexual function is maintained in 34.5% and 33% of patients treated with 70Gy and 80Gy respectively [104].

1.8.3. Overtreatment

Overtreatment is a common reality in PC management and is linked to disease overdiagnosis. In a study involving a range of urologic practices and 11,892 men 92-98% patients with low-risk tumor scores were treated with surgery, radiotherapy or hormone therapy[105]. These patients would likely be candidates for active surveillance, avoiding treatment and quality-of-life altering effects.

1.8.4. Biochemical recurrence (BCR) and persistent PSA

After primary therapy patients are subject to PSA monitoring at regular intervals. The level of PSA considered treatment failure depends on the type of primary treatment. After RP the definition of BCR is a detectable or rising PSA $\geq$ 0.2ng/ml with a second confirmatory level $\geq$ 0.2ng/ml (1). It is estimated that the risk of BCR ranges from 20-40% [32, 106]. Among the patients who have BCR, around 45% recur within 2 years of RP, 77% within 5 years and 96% within 10 years [106].

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 [107].

Persistent PSA is defined as a PSA level $\geq$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. The current treatment approach is to treat men with early salvage radiotherapy and additional hormonal therapy[32, 108].

1.8.5. Salvage treatments

Therapeutic options in these patients are monitoring, salvage local procedures or ADT (continuous or intermittent). PSA monitoring can be an option in patients at low risk of BCR, unfit patients with a short life expectancy <10 years or patients unwilling to undergo salvage treatments.

1.8.5.1.Salvage RP

Salvage RP should only be considered in selected patients and in specialized centers since the rate of adverse events is high compared to primary surgery[94]. Other salvage local treatments include brachytherapy, cryoablation and high-intensity focused ultrasound. The data on these is still poor and they should only be offered in experienced centers.

It is yet unclear the optimal timing of salvage ADT and whether it should be administered in a continuous or intermittent form. Current recommendations [32] do not advise it for M0 patients with a PSADT>12months.

1.8.5.2.Salvage RT

For BCR post-radical prostatectomy salvage radiotherapy (SRT) is the recommended option. It should be initiated in patients with a rapid PSA kinetics after RP and with a PSA cut-off of 0.4 ng/mL [91]. The optimal dose is not defined but a minimum of 64 Gy to the prostatic fossa and base of seminal vesicles (depending on the pathological stage) in combination with ADT is recommended.[32] In high-risk patients, whole pelvis irradiation is recommended opposed to prostatic fossa SRT-only [92].

Salvage ADT (for the duration of ≥2 years) after SRT for BCR for high-risk patients has shown better MFS and DSS versus observation for patients with PSADT <6months [93].

The rationale for the use of SRT versus adjuvant radiotherapy is that waiting for PSA relapse is unlikely to compromise oncological outcomes, some patients can be spared of radiotherapy, and potential treatment side effects will be delayed.

1.9. Prognosis

In a study including 9733 men with low risk and intermediate risk PC [109]on active surveillance, the 10-year cumulative incidence of metastasis was 1.5% for low-risk patients, 9.6% for favorable intermediate-risk patients, and 19.2% for unfavorable intermediate-risk patients (at least 2 intermediate-risk features and/or a Gleason score 4+3). The 10-year incidence of PCSM was 1.1% for low-risk, 3.7% for favorable intermediate-risk, and 11.8% for unfavorable intermediate-risk patients. The 10-year cumulative incidences of overall mortality were 23.2%, 26.2%, and 40.6% for the low-risk, favorable intermediate-risk, and unfavorable intermediate-risk groups, respectively. When managed with non-curative intent high-risk PC is associated with 10-year PCSM rates of 28,8%[32].

Genetic tests such as Oncotype DX GPS (prediction of adverse pathology after RP), Prolaris® (prediction of 10 year-BCR risk after RP), Decipher® (prediction of metastasis and PCSM), PORTOS (selection of patients for adjuvant RT) and ProMark Proteomic Prognostic Test (prediction of PC aggressiveness) are still investigational.

2. AIMS OF THE WORK AND HYPOTHESIS

Our work aims essentially at studying novel markers that can be used in PC diagnosis, prognosis and follow up and analyzing the role of PSA kinetics after primary treatment.

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

EN2 is a promising albeit understudied marker of PC due to exclusive secretion by PC cells, association to tumor volume and stage, simple sampling, stability and noninvasive collection from spontaneous urine without the need of previous prostate manipulation. The aim of this work was to test this marker in a more complex manner than previously done including a control group, multi-brand ELISA and prostate manipulation. Hypothesis: EN2 can be used in PC diagnosis 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 of BCR after RP which results in 'adjuvant treatment to all' with radiotherapy approach. This leads to unnecessary adverse effects to some patients. According to current recommendations the first PSA sample after surgery should be performed at 6-8 weeks, and the value at 3months used to decide on further treatment. 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 ([–2]proPSA, PHI, 4K panel) in the diagnostic setting of PC although studies as early 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 and assess their ability to predict BCR and identify disease persistence. Hypothesis: Isoforms of PSA outperform conventional PSA in predicting BCR.

3. MATERIALS AND METHODS

The material and methodology of individual experiments are described in detail in attached articles.

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

3.1.1. Study group characteristics

This work consisted of the analysis of morning urine samples of two patient groups. The first group included 90 patients with clinically localized PC before RP and a control group of 30 healthy volunteers older than 50 years of age with a negative oncologic history and screening. The second group included 40 patients indicated for prostate biopsy on the basis of elevated PSA. In the latter, pre and post-DRE urine samples were obtained.
Tab. 5 shows the different group characteristics.

Table 5: Main characteristics of analyzed study groups. Data presented as number (%) or median value (range).

Patients with Prostate cancer (n=90)	Clinical stage (cT)	T1 T2 T3	70 (78%) 15 (17%) 5 (5%)
	Pathologic stage (pT)	pT0 pT1 pT2 pT3	3 (3%) 1 (1%) 57 (64%) 29 (32%)
	Pathological Gleason score (GS)	≤7 >7	79 (91%) 8 (9%)
	Extraprostatic extension	29 (33%)	
	Seminal vesicle invasion	7 (8%)	
	Positive surgical margins (PSM)	8 (9%)	
	Biochemical recurrence (BCR)	28 (32%)	
	Age, years	66 (41-78)	
	PSA level (µg/l)	8,1 (2,7-31,8)	
	Total prostate weight (g)	44 (17-158)	
Control group of healthy volunteers (n=30)	Age, years	57,5 (51-73)	
	PSA level (µg/l)	1,05 (0,29-2,34)	
	Total prostate volume (cm^3)	25,0 (17-78)	
Patients with suspicion of prostate cancer due to elevated PSA (n=40)	Age, years	63,5 (48-77)	
	PSA level (µg/l)	7,30 (3,1-23,5)	
	Total prostate volume (cm^3)	48 (19-155)	
	Total number of biopsy cores	14 (12-20)	

3.1.2. Biochemical analysis

Urine samples were stored in 1.8ml aliquots at -76ºC and their supernatants analyzed using 3 different enzyme-linked immunoassays (Cloud-Clone Corp, Katy, TX, USA; Cusabio Biotech Co., LTD., Houston, TX, USA and MyBiosouce, Inc., San Diego, CA, USA). Several measurements were performed for each sample 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 also performed.

3.1.3. Statistical analysis

Correlations were determined using Spearman's rank correlation coefficient. The Mann–Whitney U test was used to compare the PC 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

3.2.1. Study group characteristics

A total of 406 patients from department's tumor registry with a minimum follow up of 24 months who had adverse pathology after RP (PSMs, extracapsular extension and/or seminal vesicle invasion) were initially 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. Tab. 6 shows the different group characteristics.

Table 6 Main characteristics of the analyzed study group stratified according to the occurrence of BCR. Data are shown as n, median (range) or n (%).

Variable	Overall	BCR	BCR-free	p-value
n	205	106	99	
Age at surgery (years)	65 (48–80)	64.5 (49–78)	66 (48–80)	0.315
Preoperative PSA (ng/ml)	7.4 (2.5–59.7)	8.6 (2.8–45.7)	6.6 (2.5–59.7)	0.001
PSA nadir (ng/ml)	0.021 (0.003–0.098)	0.032 (0.003–0.098)	0.010 (0.003–0.060)	0.001
Clinical stage T1 T2 T3	 129 (63) 66 (32) 10 (5)	 66 (62) 38 (36) 3 (2)	 63 (64) 28 (29) 7 (7)	0.328
Pathological Gleason sum ≤7 >7	 175 (85) 30 (15)	 93 (88) 13 (12)	 82 (83) 17 (17)	0.334
Extraprostatic extension	113 (55)	54 (51)	59 (60)	0.764
Seminal vesicle invasion	32 (16)	20 (19)	12 (12)	0.184
Positive surgical margin	97 (47)	56 (53)	41 (41)	0.102

3.2.2. Biochemical analysis

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.

3.2.3. Statistical analysis

Variables between groups of patients were compared with the Mann–Whitney test and chisquared test. BCR-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 particular PSA measurements

in predicting BCR post-RP. 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

3.3.1. Study group characteristics

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 RP without previous prostatic manipulation. When BCR or disease persistence occurred, patient follow up was ended. No patient received preoperative or postoperative hormonal androgen deprivation therapy or 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%). Tab. 7 shows the different group characteristics.

Table 7: Main patient characteristics. All the continuous variables are expressed as median (range) and categorical estimates as number (percentage).

Variable	
Age at diagnosis, years	
Median	65.5
IQR	9.0
PSA at diagnosis, ng/ml	
Median	7.8
IQR	4.9
Prostate mass, g	
Median	42.0
IQR	21.5
DRE, n (%)	
Negative	100 (78.1)
Positive	28 (21.9)
No. of patients with clinical stage (%)	100 (78.1)
cT1c	8 (6.3)
cT2a	10 (7.8)
cT2b	5 (3.9)
cT2c	6 (4.7)
cT3	
Gleason score at biopsy, n (%)	
6	89 (69.5)
3+4	17 (13.3)
4+3	12 (9.4)
8	7 (5.5)
9	2 (1.6)
Gleason score of final specimen, n (%)	
6	59 (46.1)
3+4	41 (32.0)
4+3	17 (13.3)
8	6 (4.7)
9	4 (3.1)
NA	1 (0.8)
Margins, n (%)	
Positive	34 (26.6)
Negative	94 (73.4)
Capsule involvement, n (%)	
Negative	86 (67.2)
Positive	42 (32.8)
N/A	1 (0.8)
Perineural invasion, n (%)	
Positive	79 (61.7)
Negative	47 (36.7)
N/A	2 (1.6)

Pathological stage, n (%)	
pT0	1 (0.8)
pT2	85 (66.4)
pT3a	31 (24.2)
pT3b	11 (8.6)
Lymph node status, n (%)	
pNX	78 (60.9)
pN0	47 (36.7)
pN+	3 (2.3)

3.3.2. Biochemical analysis

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 DP was defined as PSA≥0.1 ng/ml at 6 weeks after RP.

3.3.3. Statistical analysis

Variables of our 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

The results of the work are divided into subsections. There is for each subsection an original article attached.

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

4.1.1. Urinary EN2 in patients with PC versus controls

There was no statistically significant difference between the EN2 urine levels of the patient and the control group (Tab.8) and there was a pronounced difference between the EN2 levels measured by the three assays used (Kruskal–Wallis p-value <0.0001). Normalization of the EN2 level to urinary creatinine showed similar statistical distributions.

Table 8:. EN2 levels in the three assays presented as median value (range) in patients with PC and control group.

	Patient group	**Control group**	**P-value**
n	**90**	**30**	
EN2 level kit 1 (ng/ml)	5,23 (1,35-9,38)	6,22 (2,31-9,31)	0,07
EN2 level kit1/ Urine creatinine (ng/µmol)	0,58 (0,06-2,99)	0,58 (0,12-4,42)	0,85
EN2 level kit 2 (ng/ml)	0 (0-10,5)	0 (0-2,14)	0,77
EN2 level kit 2/ Urine creatinine (ng/µmol)	0 (0-3,36)	0 (0-0,19)	0,78
EN2 level kit 3 (ng/ml)	0,83 (0,15-2,47)	1,28 (0,22-2,74)	0,02
EN2 level kit3/ Urine creatinine (ng/µmol)	0,10 (0,01-0,96)	0,11 (0,01-0,92)	0,20

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 13) and no significant correlation between urine EN2 and age, tumor stage (Fig 14) or grade (Fig 15) was found.

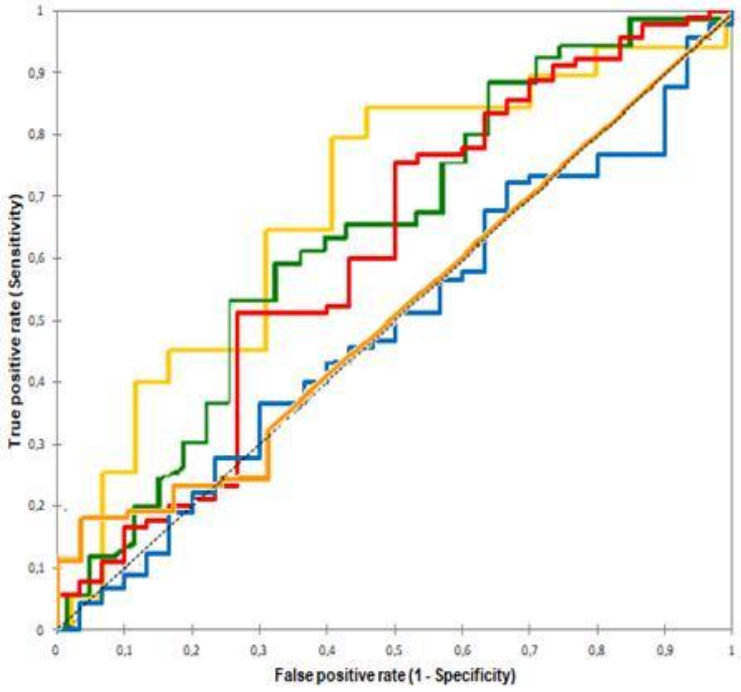

Fig. 13: Comparison of the ROC curves obtained for EN2 measured with kits 1, 2 and 3 and the ROC curve for PSA. Red: ROC curve for EN2-kit1, AUC= 0,61, Blue: ROC curve for EN2/urine Creatinine-kit1, AUC =0,49, Orange: ROC curve for EN2-kit2, AUC= 0,52, Green: ROC curve for EN2-kit3, AUC=0,65, Yellow: ROC curve for PSA, AUC=0,70

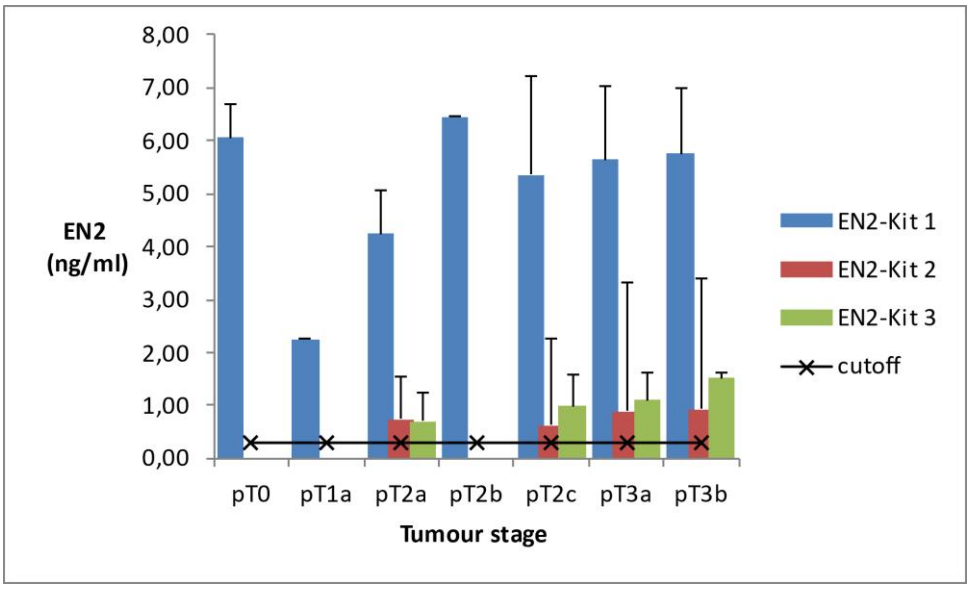

Fig. 14: Relationship between urinary EN2 and tumour stage.

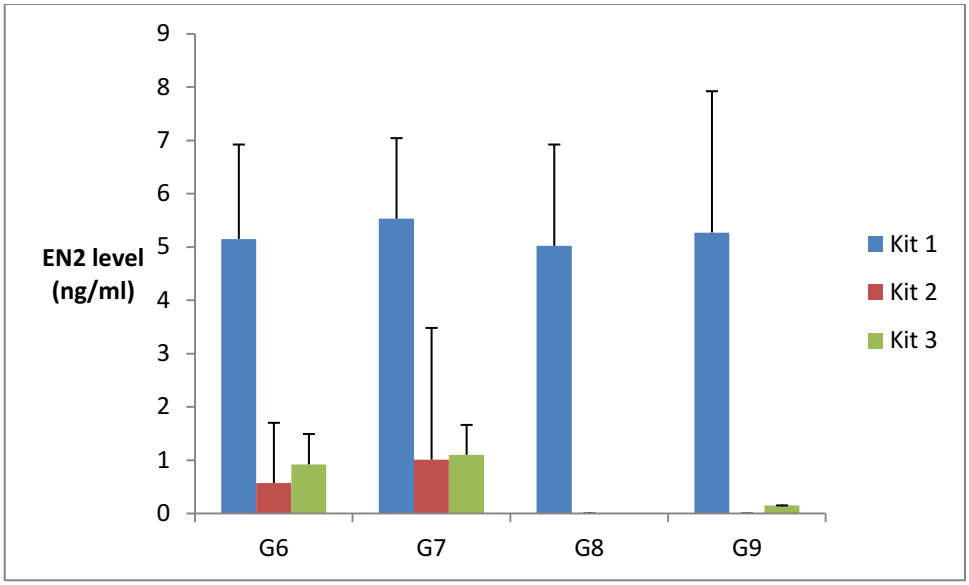

Fig . 15: Relationship between urinary EN2 and tumor Gleason grade.

4.1.2. Urinary EN2 pre- and post-DRE in patients indicated for prostate biopsy

The levels of EN2 were lower after DRE (median 1.79 ng/ml; range 0.12–5.01 ng/ml) compared to before DRE (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) (Fig. 16). 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 change the statistical distributions significantly.

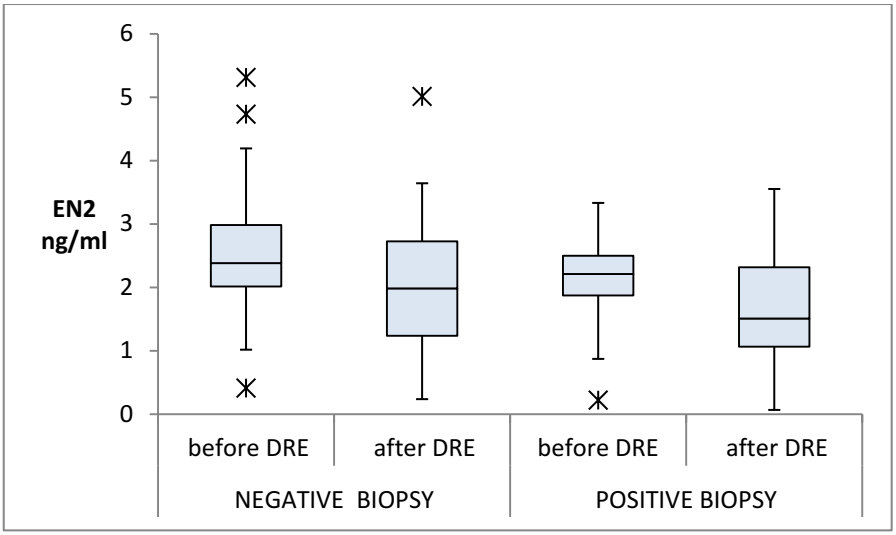

Fig. 16: EN2 concentrations in urine before and after DRE

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%), PSMs 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 BCR was 15 months (range 2–105 months). Only five men had the combination of all the adverse pathological features (PSM, seminal vesicle invasion and pT3) and these patients did not experience a significantly different rate of BCR (60%) in comparison with the rest of the cohort (51.5%, p=0.707). A similar frequency of BCR (54%) was found in men with a Gleason score higher than 7 together with PSMs 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 17 shows the ROC curves used in BCR prediction at different time points.

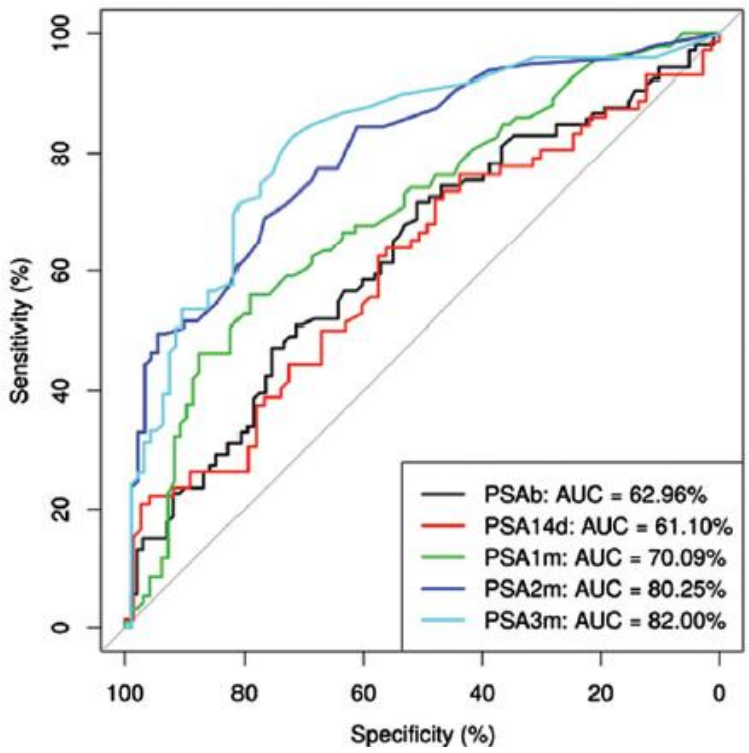

Fig. 17: ROC curves and calculated AUC values for preoperative (baseline) prostate-specific antigen (PSAb) level and postoperative PSA levels at time-points after surgery: 14 days, and 1, 2 and 3 months.

As can be seen in Fig 17 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 ($p=0.694$).

Except for PSA, none of the postoperative parameters proved to be an independent predictor of BCR in this high-risk group of patients at multivariate analysis.

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. 18).

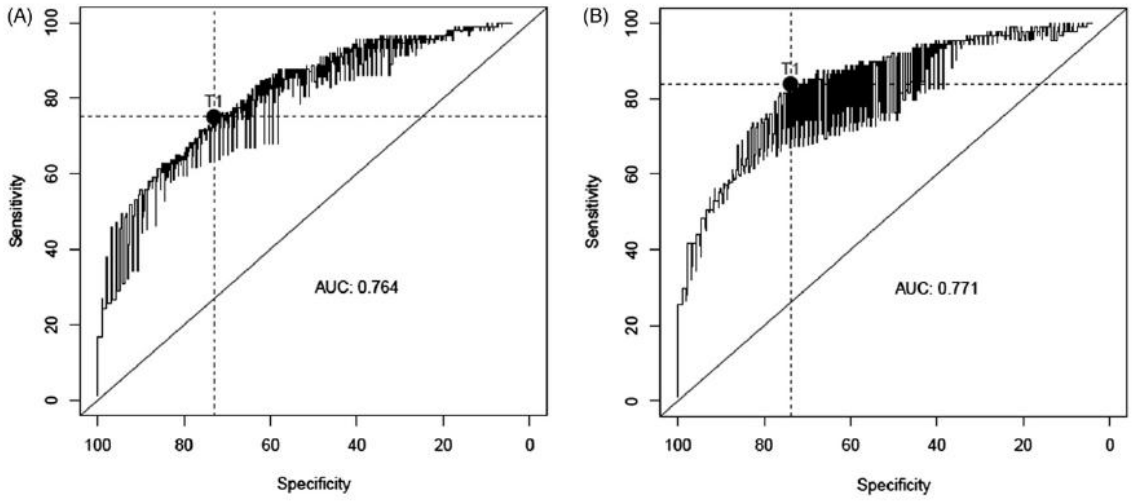

Fig. 18: Results of the sequential decision calculating the best combination of postoperative PSA cut-off levels in the model combining the PSA (A) on days 30 and 60, and (B) on days 30 and 90.

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. Fig 19 shows the stratification of patients to ART based on PSA at day 30 and day 60 after RP.

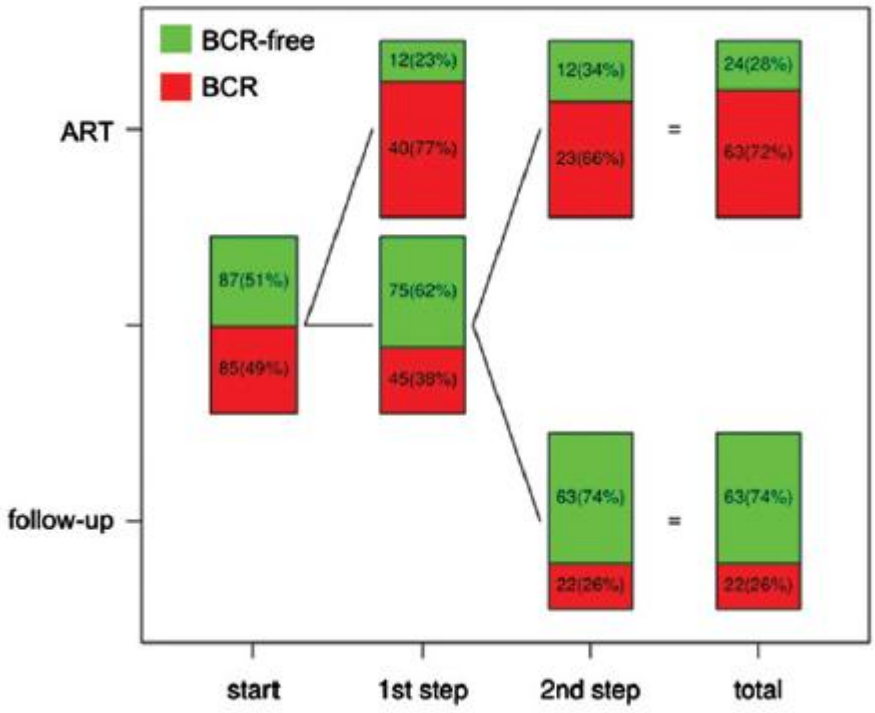

Fig. 19: Stratification of potential candidates for adjuvant radiotherapy (ART) based on PSA on day 30 (1st step) and PSA on day 60 (2nd step) and the proportion of patients with BCR in selected groups.

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 (Fig. 20). The median follow-up was 64 months. A total of 87 patients were BCR-free while 26 patients had BCR (20.3%) and 15 patients had disease persistence (11.7%).

	BCR-free group ($n=87$)	BCR-group ($n=26$)	p	DP-group ($n=15$)	p
PSA, ng/ml					
At diagnosis	7.40 (1.77–25.0)	7.75 (2.47–22.73)	≥ 0.05	10.25 (4.13–75.20)	≥ 0.05
preoperative	7.45 (1.1–28.9)	9.54 (2.86–25.28)	0.03	10.99 (3.93–84.01)	0.03
1 month	0.03 (0–0.09)	0.050 (0.010–0.2)	0.005	0.44 (0.060–1.33)	<0.0001
3 months	0 (0–0.11)	0.025 (0–0.120)	0.0004	0.485 (0.1–1.66)	<0.0001
fPSA, ng/ml					
At diagnosis	0.87 (0.29–3.13)	0.91 (0.41–1.60)	≥ 0.05	1.1 (0.54–4.23)	≥ 0.05
preoperative	0.83 (0.20–4.58)	0.955 (0.240–2.830)	≥ 0.05	1.056 (0.46–4.02)	≥ 0.05
1 month	0.010 (0–0.02)	0.010 (0–0.020)	≥ 0.05	0.030 (0.010–0.18)	<0.0001
3 months	0.01 (0–0.07)	0.010 (0–0.04)	≥ 0.05	0.060 (0.02–0.16)	<0.0001
fPSA/PSA (%fPSA)					
At diagnosis	0.11 (0.05–0.30)	0.13 (0.060–0.219)	≥ 0.05	0.0899 (0.246–0.019)	≥ 0.05
preoperative	0.12 (0.03–0.27)	0.105 (0.054–0.188)	≥ 0.05	0.115 (0.043–0.359)	≥ 0.05
1 month	0.20 (0–1.0)	0.111 (0–1.00)	≥ 0.05	0.091 (0.023–0.33)	≥ 0.05
3 months	0.50 (0–2.0)	0.200 (0–1.00)	≥ 0.05	0.142 (0.043–0.429)	≥ 0.05
[–2]proPSA, pg/ml					
preoperative	14.6 (4.19–93.3)	25.26 (6.41–85.00)	0.002	25.63 (11.12–122.5)	0.006
1 month	0.60 (0–27.0)	0.760 (0–8.56)	≥ 0.05	2.16 (0–22.64)	0.001
3 months	0.59 (0–45.6)	0.45 (0–26.57)	≥ 0.05	4.285 (0.89–20.81)	<0.0001
PHI					
preoperative	48.8 (18.2–135.1)	75.09 (25.45–195.9)	0.0003	79.89 (37.03–279.3)	0.002
1 month	13.6 (0–4789.0)	18.78 (0–1527.57)	≥ 0.05	32.15 (0–648.5)	0.04
3 months	3.46 (0–2034.6)	8.895 (0–251.37)	≥ 0.05	32.87 (14.47–382.3)	<0.0001
hK2, pg/ml					
preoperative	273.0 (0–6450.9)	180.7 (4.5–635.3)	≥ 0.05	150.8 (30.1–519.0)	≥ 0.05
1 month	308.2 (0–7435.4)	345.2 (0–1704.0)	≥ 0.05	292.6 (28.5–622.0)	≥ 0.05
3 months	339.0 (0–1426.1)	240.0 (4.5–764.8)	≥ 0.05	235.7 (22.5–647.0)	≥ 0.05

Fig. 20: Marker levels at diagnosis, preoperative, 1-month and 3-month post-RP and p-values. All the results are expressed as a median (range).

In the preoperative setting, the ability of PSA to predict BCR (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. 21). [–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 DP.

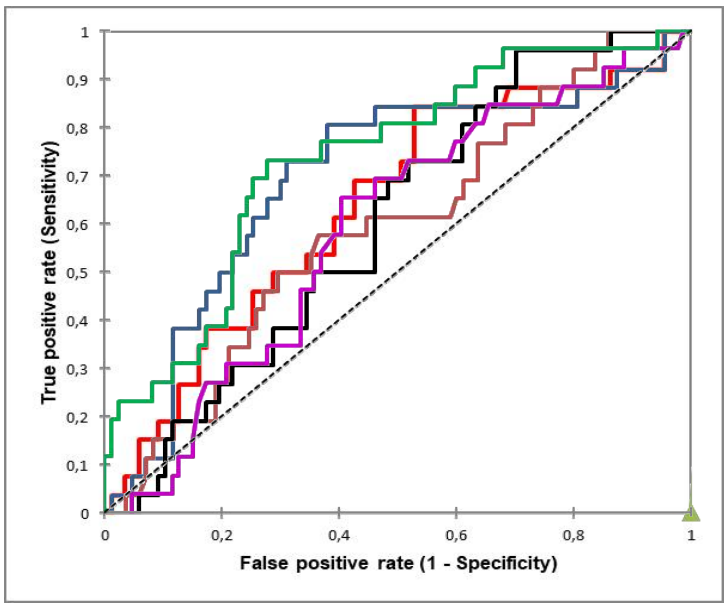

Fig. 21: ROC curves for all the markers at preoperative period and their relation to BCR. Red PSA(AUC0.64), Blue [–2]proPSA (AUC 0.70), Green PHI (AUC 0.74), Brown hK2 (AUC 0.60), Violet fPSA (AUC 0.59), Black fPSA/PSA (AUC 0.60).

In the postoperative period, PSA was the only marker that correlated with BCR at one and three months and all other markers were devoid of value (Fig.22, Fig 23)

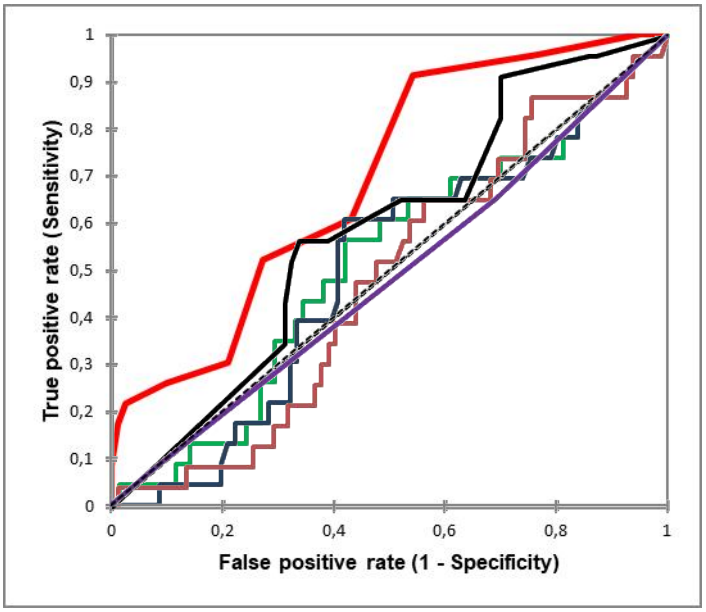

Fig. 22: ROC curves for all the markers at 1 month period and their relation to BCR. Red PSA (AUC 0.69), Blue [–2]proPSA (AUC 0.50), Green PHI (AUC 0.52), Brown hK2 (AUC 0.48), Violet fPSA (AUC 0.48), Black fPSA/PSA (AUC 0.59).

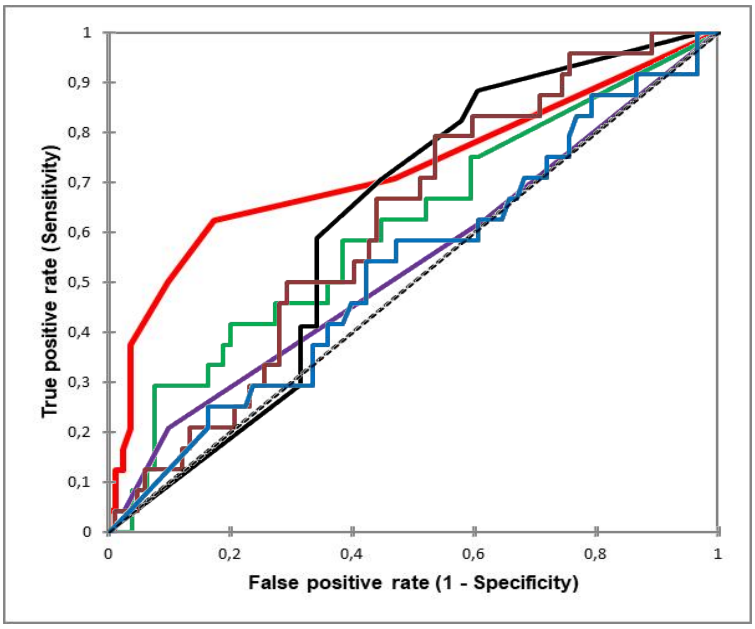

Fig. 23: ROC curves for all the markers at 3 months period and their relation to BCR. Red PSA (AUC 0.72), Blue [–2]proPSA (AUC 0.53), Green PHI (AUC 0.61), Brown hK2 (AUC 0.62), Violet fPSA (AUC 0.54), Black fPSA/PSA (AUC 0.62).

Multivariate models using preoperative data were created (Tab. 9) 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 9: 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. Regarding early post-RP identification of DP, PSA, fPSA, [–2]proPSA and PHI were all statistically significant markers (p-value≤0.03).

5. DISCUSSION

Overall, the work we developed on prostate cancer biomarkers was able to answer some questions and lead the way for further work. Initially we directed our attention to improving the diagnosis of PC. With this in mind, and after a careful search of the available literature the decision to study in depth a relatively unknown marker became attractive. The ease of collection and sample processing, low cost with possibility of wide availability, high specificity and sensitivity for PC detection were our main requisites. Engrailed-2 (EN-2) belongs to the homeobox genes (regulatory genes of embryonic development in humans and animals) and is fundamental for Purkinje cell maturation and normal cerebellar development. Diseases associated with EN2 include autistic spectrum disorders[110, 111]. Based on previous works on this marker[112–114] we designed our study. At first we included a group of 90 patients with clinically localized prostate cancer versus a group of 30 men over 50 years old with a negative oncologic screening. In contrast to other urinary markers such as PCA3, no previous prostate manipulation was deemed necessary so morning urine samples without previous prostatic massage were used. EN2 was then determined by ELISA assays, similarly to previous works. At first we used one particular assay, but since the negative results obtained were underwhelming we opted to analyze further with two other different brand assays. We did not find any statistically significant difference between the PC group and the healthy control group in any of the assays used, even after normalization to urine creatinine (Table 8). No significant correlations were found to patient (age, PSA) and tumor characteristics such as grade, stage, and tumour volume. Furthermore, there was a high discrepancy of the EN2 levels obtained by the different brand assays for both groups of patients ($p < 0.0001$). Our results oppose those of Morgan et al. (the first experimental work on EN2 as a marker of PC) published in 2011[112] and those of Pandha et al [113] published the following year in the same institution. In the pilot study 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 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 [114] 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. These results were surprising and influenced our course of action. 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). Urine samples were collected before and after DRE in both groups. No significant difference was found between the biopsy-positive and biopsy negative groups and DRE did not increase urine EN2 levels. Normalization to urine creatinine did not change these results. Our work is the most comprehensive analysis on the function of EN2 as a PC marker published till now. We designed a study including different brand ELISA assays, sample processing and examination by a single experienced operator in a blind manner, a cohort consisting of a control group, PC 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. The urinary EN2 test was licensed to Zeus Scientific as it reported in 2013 with projections of submission to USFDA in 1 year and worldwide clinical use in 2 years time[115]. 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 is not currently registered at ClinicalTrials.gov [116] .

Approximately one third of patients after RP performed for clinically localized PC will have adverse pathological features in the form of PSM, EPE, or SVI [117] . This patient group considered at high risk for BCR is very heterogenous and their clinical course is uncertain. The current indication for ART in clinically localized PC is for patients with ISUP 4-5 and pT3 ± PSM [62]. Overtreatment with ART is a reality that can affect 35-60% of these high risk patients [118] besides possible adverse events including genitourinary 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 RP. Ultrasensitive PSA tests were 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% at the cost of undertreating 22 patients (13%). Out of these, 18 would have progression on day 90 and another two patients on day 120, and only 2 patients (1.17%) would stay undertreated with late appearance of BCR. The results obtained are in line with previous studies by Audenet et al [119] and Shen et al [120] 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 [120]**.** Eisenberg et al [121] showed that men with undetectable ultrasensitive PSA<0.01 ng/ml have a low risk of BCR 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 ‘ART 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 ART-treated arm. Prospective studies with ultrasensitive PSA are needed to further determine which patients will benefit from adjuvant therapy and which patients can be spared.

With a sustained interest on improving the prediction of BCR pre- and postoperatively we designed a third study including 128 patients who underwent RP. Blood samples were collected before surgery, and the follow up (median 64 months) included DRE and blood samples at 1 and 3 months after surgery. PSA, fPSA, [–2]proPSA and hk2 were the markers selected for analysis. The preoperative predictors of BCR 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 was in line with previous works by Lughezzani et al. [122] and Maxeiner et al. [123] 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. [124] 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 [125] 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 BCR is still budding and the available preoperative nomograms such as the MSKCC [126] or CAPRA score [127] 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. We hypothesize that cross-validation of models including [–2]proPSA and especially PHI in preoperative nomograms, on a larger population, in a prospective and multicentre setting would be of a great value.

6. CONCLUSION

Urinary EN2 tests were reported to be easy, non-invasive, inexpensive with high sensitivity and specificity to detect PC and their commercial use was predicted to start in 2015. External and larger scale validation studies were lacking. We provided the most comprehensive analysis of this marker to date and our results point to its inability to detect PC in contrast to what was previously thought. We found no difference between EN2 levels of patients and controls, a great dependency of EN2 on the type of assay used, no relevant association with clinicopathological characteristics and no improved value following prostate manipulation or urine creatinine normalization. Thus, our study discourages further investigation on this marker.

With the knowledge that BCR precedes the occurrence of metastases with a mean time of 8 years and PCSM with a mean time of 15 years, and with the objective to better stratify high-risk patients to adjuvant treatment and minimize overtreatment, we turned our attention to early prediction of BCR. We determined that the sampling of ultrasensitive PSA as early as day 30 after RP strongly correlated to BCR and its use in stratification models for high-risk patients can reduce overtreatment as much as 37% in this population group.

With a maintained interest in disease prognosis and prediction of BCR we decided to analyze isoforms of serum PSA which have been clinically used in PC diagnosis and at decision making to perform biopsy. We concluded that [–2]proPSA and PHI clearly outperform serum PSA as predictors of BCR and disease persistence preoperatively. On the other hand the postoperative use of these isoforms is devoid of value. This is of consequence regarding patient information, surgery planning, individualization of follow up and adjuvant treatment strategy. The inclusion of these isoforms in preoperative nomograms instead of serum PSA seems a logical step and altogether combination with early sampling of ultrasensitive PSA after RP could bring more individualized care, minimizing overtreatment.

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 such as bone pain caused by metastases. 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 has inherent limitations such as fluctuations with age, prostate size, inflammation and infection, recent prostate manipulation, ejaculation and some medicaments. So far and despite these limitations no marker has been able to replace serum prostate specific antigen 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 new and relatively unknown non-invasive urinary marker advocated to have high detection sensitivity and specificity, to explore the early postoperative prostate specific antigen fluctuations and their relation to the occurrence of biochemical recurrence in a population at high risk and to study the role of prostate specific antigen isoforms in predicting biochemical recurrence and disease persistence before and after radical prostatectomy. Our 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 ultrasensitive 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 radiotherapy in this patient group. The latter study showed 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 current marker of choice. The use of [–2]proPSA and PHI in preoperative nomograms instead of prostate specific antigen seems the logical next step.

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9. LIST OF FIGURES AND TABLES

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