Turun yliopisto
University of Turku

IDENTIFICATION AND VALIDATION OF NOVEL PROSTATE CANCER BIOMARKERS

Saeid Alinezhad

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
Sarja - ser. A II osa - tom. 312 | Biologica - Geographica - Geologica | Turku 2016

University of Turku

Faculty of Mathematics and Natural Sciences
Department of Biochemistry
Molecular Biotechnology and Diagnostics
Doctoral Programme in Molecular Life Sciences

Supervised by

Professor Kim Pettersson
Department of Biochemistry
Molecular Biotechnology and Diagnostics
University of Turku
Turku, Finland

Riina-Minna Väänänen, Ph. D.
Department of Biochemistry
Molecular Biotechnology and Diagnostics
University of Turku
Turku, Finland

Reviewed by

Professor Craig Robson, Ph. D.
Northern Institute for Cancer Research
Newcastle University Medical School
Newcastel, UK

Dr. Gerald W. Verhaegh, Ph. D.
Radboud Institute for Molecular Life Sciences
Radboud University Medical Center
Nijmegen, The Netherlands

Opponent

Professor Guido Jenster, Ph. D.
Josephine Nefkens Institute
Department of Urology
Erasmus Medical Center
Rotterdam, The Netherlands

Cover image by Saeid Alinezhad

The originality of this thesis has been checked in accordance with the University of Turku quality assurance system using the Turnitin OriginalityCheck service.

ISBN 978-951-29-6419-2 (PRINT)
ISBN 978-951-29-6420-8 (PDF)
ISSN 0082-6979 (PRINT)
ISSN 2343-3183 (ONLINE)
Painosalama Oy - Turku, Finland 2016

To my parents

TABLE OF CONTENTS

LIST OF ORIGINAL PUBLICATIONS 6

ABBREVIATIONS 7

ABSTRACT 9

TIIVISTELMÄ 10

1. LITERATURE REVIEW 11

1.1. Normal prostate gland 11
1.2. Prostate diseases 11
1.2.1. Prostatitis 11
1.2.2. Benign prostate hyperplasia (BPH) 11
1.2.3. Prostate cancer (PCa) 12
1.2.3.1. Risk factors for PCa 12
1.2.3.2. Development of PCa 12
1.2.3.3. Histology and grading of PCa 13
1.2.3.4. Clinical management of PCa 16
1.2.3.5. AR and castration resistant prostate cancer (CRPC) 18
1.3. Diagnosis of PCa 19
1.3.1. Different biomarkers for PCa 20
1.3.2. Types of biological samples available for identification of PCa biomarkers 22
1.3.3. Protein-based biomarkers 23
1.3.4. DNA-based biomarkers 23
1.3.5. RNA-based biomarkers 25
1.3.5.1. miRNAs in PCa 25
1.3.5.2. Long non-coding RNAs in PCa 26
1.3.5.3. mRNA transcripts as PCa biomarkers 26
1.4. Microarray and gene expression profiling 27
1.5. DNA and RNA sequencing 28
1.6. Real-time PCR methods 28
1.6.1. Different quantification strategies 29
1.6.2. Normalization of RT-PCR 29
1.6.3. Detection chemistries 30

2. AIMS OF THE STUDY 31

3. SUMMARY OF MATERIALS AND METHODS 32

3.1. Clinical samples 32
3.1.1. Prostate tissues from patients with PCa (Cohort A) (I,II)......................32
3.1.2. Prostate tissues from patients with PCa (Cohort B) (III).......................33
3.1.3. Prostate tissue from cross-section of PCa patients (II) 33
3.1.4. Prostate tissues from men with bladder cancer (II, III)..........................33
3.1.5. Prostate tissue biopsies from men with clinical suspicion of PCa (IV) 34
3.1.6. Blood samples from PCa patients and healthy individuals (I).............34
3.2. RNA extraction and reverse transcription 34
3.3. Internal and external RNA standards (I, II, III, IV) 35
3.4. Real-time PCR 35
3.4.1. Reagents and temperature profile conditions..36
3.5. Immunohistochemistry (II) 37
3.6. Gene knock-down using RNA interference 40
3.7. Cell migration and invasion assay (wound healing) 40
3.8. 3D cell culture and image acquisition for morphological analyses.................40
3.9. Data analyses...40
3.10. Statistical analyses 41

4. RESULTS AND DISCUSSION...42

4.1. Lanthanide chelates and TRF in RT-PCR (I)..42
4.2. Expression of *AMACR* and *AR* in cancerous and noncancerous prostate (II)..44
4.2.1. Overexpression of *AMACR*...44
4.2.2. Overexpression of *AR* and *KLK3*...45
4.3. Identification and validation of novel biomarkers for PCa (III) 46
4.3.1. Evaluation of the mRNA expression of candidate genes in tissue samples 46
4.3.2. Knockdown with siRNA transfection and its effects on invasion and motility of PC-3 cells...50
4.3.3. Effect of gene knockdown on PC-3 cell morphology in 3D model......50
4.4. Expression of 11 biomarker genes in apparently benign tissue samples (IV)..52

5. CONCLUSIONS...56

AKNOWLEDGMENTS...58

REFERENCES 60

ORIGINAL PUBLICATIONS 69

LIST OF ORIGINAL PUBLICATIONS

I. Alinezhad S, Väänänen RM, Lehmusvuori A, Karhunen U, Soukka T, Kahkonen E, Taimen P, Alanen K, Pettersson K: Lanthanide chelate complementation and hydrolysis enhanced luminescent chelate in real-time reverse transcription polymerase chain reaction assays for KLK3 transcripts. *Analytical Biochemistry* 2014, 444:1-7

II. Alinezhad S, Väänänen RM, Tong Ochoa N, Vertosick EA, Bjartell A, Boström PJ, Taimen P, Pettersson K: Global expression of AMACR transcripts predicts risk for prostate cancer – a systematic comparison of AMACR protein and mRNA expression in cancerous and noncancerous prostate. *BMC Urology* (2016) 16:10.

III. Alinezhad S, Väänänen RM, Mattsson J, Li Y, Tallgrén T, Tong Ochoa N, Bjartell A, Åkerfelt M, Taimen P, Boström PJ, Pettersson K, Nees M: Identification of novel biomarkers for prostate cancer progression based on the combination of bioinformatics, functional and clinical validation. *Submitted manuscript.*

IV. Alinezhad S, Väänänen RM, Tallgrén T, Perez IM, Jambor I, Aronen H, Kähkönen E, Ettala O, Syvänen K, Nees M, Kallajoki M, Taimen P, Boström PJ, Pettersson K: Stratification of aggressive prostate cancer from indolent disease – prospective controlled trial utilizing expression of 11 genes in apparently benign tissue. *Urologic Oncology*, 2016.

ABBREVIATIONS

ACSM1	acyl-coA synthetase medium-chain family member 1
AMACR	alpha-methyl Co-A racemase
AR	androgen receptor
BPH	benign prostate hyperplasia
CACNA1D	calcium channel, voltage-dependent, L-type, alpha 1D subunit
CP	cystoprostatectomy
CRPC	castration-resistant prostate cancer
CTC	circulating tumor cells
DLX1	distal-less homeobox 1
DRE	digital rectal examination
dsDNA	double-stranded DNA
ELISA	enzyme-linked immunosorbent assays
EPCA	early prostate cancer antigen
ESTs	expressed sequence tags
FRET	fluorescence resonance energy transfer
HELC	hydrolysis enhanced luminescent chelate
HGPIN	high-grade PIN
KLK2	kallikrein-related peptidase 2
KLK3	kallikrein-related peptidase 3
LCC	lanthanide chelate complementation
LGPIN	low-grade PIN
LMNB1	lamin B1
lnRNA	long non-coding RNA
LPOCV	leave-pair-out cross-validation
miRNA	micro RNA
NGS	next-generation sequencing
PCa	prostate cancer
PCA3	prostate cancer antigen 3
PCR	polymerase chain reactions
PIN	prostatic intraepithelial neoplasia

PLA2G7	phospholipase A2, group VII
PRNCR1	PCa noncoding RNA 1
PSA	prostate-specific antigen
PSCA	prostate stem cell antigen
PSMA	prostate-specific membrane antigen
PTEN	phosphatase and tensin homolog
qRT-PCR	quantitative reverse transcription polymerase chain reactions
RB	retinobelastoma
RHOU	ras homolog family member U
RP	radical prostatectomy
RT-PCR	reverse transcription polymerase chain reactions
siRNA	small interfering RNA
SPINK1	serine peptidase inhibitor, Kazal type 1
SPON2	spondin 2
TDRD1	tudor domain containing 1
TMPRSS2-ERG	transmembrane protease, serine 2 and etv-related gene fusion

ABSTRACT

Prostate cancer (PCa) has emerged as the most commonly diagnosed lethal cancer in European men. PCa is a heterogeneous cancer that in the majority of the cases is slow growing: consequently, these patients would not need any medical treatment. Current diagnostic methods of PCa suffer from a lack of sensitivity and specificity that may cause either missed cancers or overtreatment as a consequence of over-diagnosis. Therefore, more reliable biomarkers are needed for a better discrimination between indolent and potentially aggressive cancers.

The aim of this thesis was the identification and validation of novel biomarkers for PCa. The mRNA expression level of 14 genes including *AMACR, AR, PCA3, SPINK1, TMPRSS2-ERG, KLK3, ACSM1, CACNA1D, DLX1, LMNB1, PLA2G7, RHOU, SPON2*, and *TDRD1* was measured by an absolutly quantitative reverse transcription PCR in prostate tissue samples from men with and without PCa. For the last eight genes, the function in PCa progression was studied by a specific siRNA knockdown in PC-3 and VCaP cells.

The results showed statistically significant overexpression for all the target genes, except for *KLK3* in men with PCa compared with men without PCa. Interestingly, *AMACR* mRNA expression was similarly upregulated in all samples from men with PCa even if taken from apparently histologically benign areas; thus pointing to potential "field effects" in cancer-adjacent or associated tissues. Significant differences were observed in low versus high Gleason grades (for *PLA2G7*), PSA relapse versus no relapse (for *SPON2*), and low versus high TNM stages (for *CACNA1D* and *DLX1*). The knockdown by siRNA resulted in a cytotoxic effect for *DLX1, PLA2G7*, and *RHOU* silencing; furthermore, a cell invasion alteration was caused by *PLA2G7, RHOU, ACSM1*, and *CACNA1D* silencing in 3D. *TDRD1* and *TMPRSS2-ERG* expression was useful in discriminating between men with and without PCa even when applied to apparently benign prostate tissue biopsies from men with a clinical suspicion of PCa. Utilizing a multivariate analysis using RNA expression of 12 genes in combination with serum PSA, F/T PSA, and prostate volume outperformed each individual marker in distinguishing aggressive PCa from indolent disease. Altogether, these findings indicate the possibility of utilizing these new markers as diagnostic and prognostic markers, and they may also represent therapeutic targets for PCa.

TIIVISTELMÄ

Eturauhassyöpä on yleisin eurooppalaisilla miehillä diagnosoitu, kuolemaanjohtava syöpä. Se on heterogeeninen tauti, joka useimmissa tapauksissa etenee hitaasti eikä siten välttämättä vaadi lääketieteellistä hoitoa. Eturauhassyövän diagnosoinnissa käytetyt menetelmät eivät kuitenkaan ole tarpeeksi herkkiä ja spesifisiä, joten monet syöpätapaukset voivat jäädä huomaamatta, tai toisaalta joitakin tapauksia voidaan ylihoitaa tarpeettomasti. Luotettavampia biomerkkiaineita tarvitaan, jotta sekä hidaskasvuiset että myös mahdollisesti aggressiivisiksi osoittautuvat syövät voidaan tunnistaa.

Tämän väitöskirjatyön tavoitteena oli löytää ja validoida uusia biomerkkiaineita eturauhassyövän diagnostiikkaan. Neljäntoista geenin – *AMACR*:n*,* *AR*:n*,* *PCA3*:n*,* *SPINK1*:n*,* *TMPRSS2-ERG*:n*,* *KLK3*:n*,* *ACSM1*:n*,* *CACNA1D*:n*,* *DLX1*:n*,* *LMNB1*:n*,* *PLA2G7*:n*,* *RHOU*:n*,* *SPON2*:n ja *TDRD1*:n – lähetti-RNA-tasoja mitattiin täysin kvantitatiivisella käänteiskopiointi-PCR-määrityksellä sekä eturauhassyöpää sairastavien että sairastamattomien miesten kudosnäytteistä. Viimeksi mainittujen kahdeksan geenin osalta tutkittiin myös niiden osuutta eturauhassyövän etenemisessä vaimentamalla siRNA-molekyylien avulla niiden toimintaa PC-3- ja VCaP-soluissa.

Kaikki tutkitut geenit *KLK3*:a lukuunottamatta yli-ilmenivät tilastollisesti merkittävästi miehillä, jotka sairastivat eturauhassyöpää. Kiinnostavaa oli myös, että *AMACR*-RNA:n ilmeneminen oli lisääntynyt kaikissa eturauhassyöpäpotilaiden näytteissä – myös niissä, jotka olivat peräisin näennäisesti hyvänlaatuisilta kudosalueilta, mikä viittaisi mahdolliseen kenttävaikutukseen syöpäkudoksen läheisyydessä olevissa kudoksissa. Tilastollisesti merkittäviä ilmenemistasojen muutoksia havaittiin Gleason-asteeltaan (*PLA2G7*) tai TNM-luokitukseltaan (*CACNA1D* ja *DLX1*) korkeiksi luokitelluissa kudoksissa sekä PSA-relapsipotilaiden kudoksissa (*SPON2*). *DLX1*:llä, *PLA2G7*:lla ja *RHOU*:lla havaittiin olevan sytotoksisia vaikutuksia ja *PLA2G7*:n*,* *RHOU*:n*,* *ACSM1*:n ja *CACNA1D*:n havaittiin vaikuttavan solujen invaasiokykyyn. *TDRD1-* ja *TMPRSS2-ERG*-RNA-tasot auttoivat tunnistamaan eturauhassyöpätapauksia jopa hyvänlaatuisten eturauhaskoepalojen perusteella. Multivarianttianalyysi, joka yhdisti 12 geenin RNA-tasot seerumin PSA- ja F/T PSA- tasoihin sekä eturauhasen kokoon, tunnisti aggressiiviset syövät paremmin kuin mikään yksittäinen merkkiaine. Kaiken kaikkiaan tässä työssä tehdyt havainnot viittaavat tutkittujen geenien olevan mahdollisesti käyttökelpoisia diagnostisina ja ennustavina merkkiaineina sekä lääkekohteina eturauhassyövässä.

1. LITERATURE REVIEW

1.1. Normal prostate gland

The prostate is one of the largest glands of the human reproductive system. It is shaped like an inverted pyramid, and in adults it is approximately the size of a chestnut/walnut. The prostate is located between the bladder and penis. The urethra passes through the central part of the prostate and transports body fluids from bladder to penis. The prostate's secretions make up about 30% of seminal fluids in ejaculate, and have both protective and nourishing functions for the sperm. The prostate gland is composed of four zones (McNeal, 1968; McNeal, 1980): the transitional, central and peripheral zones as well as the anterior fibromuscular stroma. The transitional zone represents approximately 5% of the normal prostate; benign prostate hyperplasia (BPH), the benign enlargement of prostate, mainly occurs in this zone, whereas prostate adenocarcinoma is quite rare in this region. The central zone forms about one fourth of the prostate, and ejaculatory ducts are located in the middle of this zone. The peripheral zone makes up 60-70% of the prostate gland; most of prostate adenocarcinomas arise within this zone, and prostatitis is also common. The fourth zone of prostate, the anterior fibromuscular stroma, constitutes the remainder, approximately 25-30%, of the prostate gland.

1.2. Prostate diseases

1.2.1. Prostatitis

Prostatitis is an inflammation or infection of the prostate that sometimes also affects the area around the prostate. Prostatitis is the most common urinary tract problem in men below the age of 50 years, and it affects up to 15% of the male population in the United States (Murphy *et al.*, 2009). Prostatitis is classified as acute bacterial prostatitis, bacterial or chronic pelvic pain syndrome, or asymptomatic prostatitis. Prostatitis can be very painful and may negatively affect the patient's life quality.

1.2.2. Benign prostate hyperplasia (BPH)

BPH refers to the enlargement of prostate in men due to the proliferation of epithelial cells and smooth muscles within the transitional zone of the prostate. BPH is a progressive disorder, and patient age is the most important risk factor. The prevalence of BPH among men in their 40s is only about 25%, while it increases to > 90% in men older than 80 years (Sarma and Wei, 2012). BPH is not a life-threatening disease, but it can cause lower urinary tract symptoms, e.g., hesitancy (a delay between trying to urinate

and urine starting to flow), or cause frequent urination that can significantly reduce the patients' quality of life.

1.2.3. Prostate cancer (PCa)

Although adenocarcinoma of the prostate, or prostate cancer (PCa), has a much lower incidence rate than BPH, it is the most frequently diagnosed cancer type, and the second largest cause of cancer-related death in men. On the basis of a surveillance study conducted by American Cancer Society, it has been estimated that in 2015, over 200,000 new PCa cases will be diagnosed. This equals 26% of the total number of newly diagnosed cancer cases in men in the US.

In the early stages, PCa usually causes no symptoms. Nevertheless, patients with advanced PCa frequently suffer from urinary problems such as difficulty in starting or stopping the urine flow, a frequent need to urinate, painful urination and blood in the urine. Metastasized PCa commonly causes severe pain, mainly due to the spreading of cancer cells to the bone.

1.2.3.1. Risk factors for PCa

PCa is a multifactorial disease, and several genetic and non-genetic factors are involved in its initiation and progression. Age, family history and ethnicity are considered as the most important, prevalent risk factors for PCa. Aging is the predominant risk factor for PCa. On the basis of an age-specific study it has been shown that in the age-adjusted incidence curve the risk of PCa starts to rise in men above 55 years of age and it peaks at the age of 74 years, after which the statistical incidence rate begins to slightly decline (Gann, 2002). Only 10 to 20 percent of PCa cases are considered familial (Stanford and Ostrander, 2001), whereas 75 to 85 percent of PCa cases are sporadic (Stanford and Ostrander, 2001; Carter *et al.*, 1993; Ostrander, Markianos and Stanford, 2004). However, another study performed on 44,788 pairs of twins revealed a 42% risk for heritable factors for PCa (Lichtenstein *et al.*, 2000). There is also a 1.4 times higher risk of being diagnosed with PCa and two to three times higher risk of dying of the disease for African men compared to Caucasian men (Chornokur *et al.*, 2011). In addition to risk factors mentioned above, several other factors such as diet, lifestyle, environmental factors, steroid hormones and body mass index may have a role in PCa development.

1.2.3.2. Development of PCa

Carcinogenesis is considered to be a complex process arising from the accumulation of different genetic changes and requiring several steps. On the basis of this hypothesis, it could be concluded that different stages and grades of PCa may be associated with specific and unique genetic alterations. These initiating genetic and genomic instabilities

are followed by a clonal selection process involving further genetic changes and giving tumor cells more capacity for autonomy. Although several allelic losses related to PCa carcinogenesis have been reported, none of them have resulted in a conclusive designation of a single candidate tumor suppressor gene involved in the progression of PCa.

The loss of specific regions in chromosome 8p, is common in patients with colorectal and lung cancer, has also been reported in approximately 80% of patients with prostate tumors (Chang *et al.*, 1994; Fujiwara *et al.*, 1994; Matsuyama *et al.*, 1994; Imbert *et al.*, 1996; Wistuba *et al.*, 1999). Several studies have suggested that a loss of 8p1-21 locus that encodes the *NKX3.1* homeobox gene occurs in the primary precursor lesions in human PCa, prostate intraepithelial neoplasia (PIN) as well as early-stage PCa (Bhatia-Gaur *et al.*, 1999; Voeller *et al.*, 1997). A loss of *PTEN* (Phosphatase And Tensin Homolog) caused by the loss of certain regions on chromosome locus 10q-23 has been reported in 50 to 80 percent of PCa cases in several independent studies (Saric *et al.*, 1999; Ittmann, 1996; Trybus *et al.*, 1996; Cher *et al.*, 1996). A mutation of *PTEN* has also been frequently detected in metastasized PCa and, to a lesser degree, in localized PCa (McMenamin *et al.*, 1999; Suzuki *et al.*, 1998). A deletion or mutation of *PTEN* results in increased cell proliferation and reduced cell death. Retinoblastoma (*RB*) gene is another tumor suppressor for which genetic loss or mutations have been reported in about 50% of PCa patients (Cooney *et al.*, 1996; Melamed, Einhorn and Ittmann, 1997). A loss of a region of chromosome 17p, which includes the *p53* gene occurs preferentially in the advanced stages of PCa and is associated with progression to metastatic disease (Cher *et al.*, 1994; Saric *et al.*, 1999; Brooks *et al.*, 1996). It has been reported that acquisition of TP53 mutations is linked with the expansion of metastatic subclones (Hong *et al.*, 2015). Moreover, association of the amplification of MYCL in evolution of multifocal PCa has been demonstrated (Boutros *et al.*, 2015).

In addition to the deletion and mutation of tumor suppressor genes, several other genetic changes in different pathways can occur, for example, mutation, overexpression or amplification of cell cycle regulatory genes, androgen receptor signaling pathway genes, apoptotic regulatory genes and genes involved in telomerase activity. These changes are involved in the initiation and progression of prostate adenocarcinoma (Zhang *et al.*, 1998; Shen and Abate-Shen, 2010).

1.2.3.3. Histology and grading of PCa

Due to the heterogeneous and multifocal nature of PCa, its grading is a challenging issue for pathologists. A cancerous prostate normally consists of a juxtaposition of benign tissue, PIN foci and neoplastic (cancerous) foci with different grades.

PIN is identified as the primary progenitor for prostatic adenocarcinoma. PIN manifests either as low-grade (LGPIN) or high-grade (HGPIN). Since LGPIN shares common structures with normal prostate, it is not considered as PCa precursor (Bostwick, 2000). Although some studies have reported the presence of cancer in repeated biopsies for LGPIN cases (Goeman *et al.*, 2003) it has been suggested that mentioning LGPIN in diagnostic reports be avoided (Srigley *et al.*, 2000). According to several studies, HGPIN is the most frequent and most relevant precursor lesion for prostate adenocarcinoma (Singh *et al.*, 2009; Powell *et al.*, 2010; Dovey, Corbishley and Kirby, 2005; Joniau *et al.*, 2005; Vis and Van Der Kwast, 2001; Gaudin *et al.*, 1997; Pacelli and Bostwick, 1997). HGPIN exhibits several similarities with PCa, including molecular, biochemical and cytological changes (Vis and Van Der Kwast, 2001). Furthermore, increased HGPIN with age incidence, its occurrence predominantly in the peripheral zone of the prostate and pronounced multifocality are other similarities between HGPIN and PCa (Joniau *et al.*, 2005; Brawer, 2005).

As in many other diseases, histopathology is the gold standard method for the diagnosis and staging of prostate adenocarcinoma. The Gleason grading system has remained the most widely used method for prostate adenocarcinoma grading since its introduction in 1966 by Donald F. Gleason (Gleason, 1966). Gleason grading is based on the microscopic examination of glandular architecture in hematoxylin and eosin-stained histological slides (H&E stain). In this system, glandular tissue architecture is divided into five characteristic patterns or tissue architectures, based on the level of growth and differentiation that is identified by numbers from 1 (most differentiated) to 5 (the least differentiated). The final Gleason score is the sum of predominant and the second most prevalent patterns, ranging from 2 to 10.

Furthermore, TNM classification is the most widely used staging system for PCa. TNM assesses the extent of the primary tumor (T category), the extent of involved lymph nodes (N) and any distant metastasis (M). Moreover TNM staging in PCa takes the cancer grade into account (Tables 1 and 2).

Table 1. TNM classification of PCa

Category to be assessed	**Stage**	**Definition**
Primary tumor (Clinical)	TX	Primary tumor cannot be assessed
	T0	No evidence of primary tumor
	T1	Clinically inapparent tumor not palpable or visible by imaging
	T1a	Tumor incidental histologic finding in ≤5% of tissue resected
	T1b	Tumor incidental histologic finding in >5% of tissue resected
	T1c	Tumor identified by needle biopsy (because of elevated PSA level)
	T2	Tumor confined within prostate; tumors found in 1 or both lobes by needle biopsy but not palpable or reliably visible by imaging
	T2a	Tumor involves one half of 1 lobe or less
	T2b	Tumor involves more than one half of 1 lobe but not both lobes
	T2c	Tumor involves both lobes
	T3	Tumor extends through the prostatic capsule; invasion into the prostatic apex, or the prostatic capsule is classified not as T3 but as T2
	T3a	Extracapsular extension (unilateral or bilateral)
	T3b	Tumor invades seminal vesicle(s)
	T4	Tumor fixed or invades adjacent structures other than seminal vesicles (e.g., bladder, levator muscles, and/or pelvic wall)
Primary tumor (pathological)	pT2	Organ-confined
	pT2a	Unilateral, involving one half of 1 lobe or less
	pT2b	Unilateral, involving more than one half of 1 lobe but not both lobes
	pT2c	Bilateral disease
	pT3	Extraprostatic extension
	pT3a	Extraprostatic extension or microscopic invasion of the bladder neck
	pT3b	Seminal vesicle invasion
	pT4	Invasion of the bladder and rectum
Regional lymph nodes (clinical)	**NX**	Regional lymph nodes were not assessed
	N0	No regional lymph node metastasis
	N1	Metastasis in regional lymph node(s)
Regional lymph nodes (pathological)	**PNX**	Regional nodes not sampled
	pN0	No positive regional nodes
	pN1	Metastases in regional nodes(s)
Distant metastasis (M)	**M0**	No distant metastasis
	M1	Distant metastasis
	M1a	Non-regional lymph nodes(s)
	M1b	Bone(s)
	M1c	Other site(s) with or without bone disease

Table 2. Prostate cancer anatomic stage/ prognostic groups, based on the 7th edition of American Joint Committee on Cancer (2009)

Group	T	N	M	PSA ng/ml	Gleason
I	T1a–c	N0	M0	PSA <10	Gleason ≤6
	T2a	N0	M0	PSA <10	Gleason ≤6
	T1–2a	N0	M0	PSA X	Gleason X
IIA	T1a–c	N0	M0	PSA <20	Gleason 7
	T1a–c	N0	M0	PSA ≥10 <20	Gleason ≤6
	T2a	N0	M0	PSA <20	Gleason ≤7
	T2b	N0	M0	PSA <20	Gleason ≤7
	T2b	N0	M0	PSA X	Gleason X
IIB	T2c	N0	M0	Any PSA	Any Gleason
	T1–2	N0	M0	PSA ≥20	Any Gleason
	T1–2	N0	M0	Any PSA	Gleason ≥8
III	T3a–b	N0	M0	Any PSA	Any Gleason
IV	T4	N0	M0	Any PSA	Any Gleason
	Any T	N1	M0	Any PSA	Any Gleason
	Any T	Any N	M1	Any PSA	Any Gleason

1.2.3.4. Clinical management of PCa

Several treatment options are available for PCa but before any decision is made, the patient's life expectancy, his overall health status and the characteristics of tumor should be assessed. The treatment of localized PCa, which has not yet spread to other organs, is more straight forward than that of metastasized PCa. The main treatment choices for PCa are:

1- Active surveillance/watchful waiting

In some patients with low risk PCa, especially at an advanced age, the cancer may never become life threatening and aggressive therapies can be avoided. Watchful waiting which has been studied in several randomized controlled trials (Bill-Axelson *et al.*, 2011; Iversen, Madsen and Corle, 1995; Wilt and Brawer, 1994), requires regular check-ups where the time between visits varies from patient to patient. If the cancer shows any signs of growth or progression to more aggressive stages, additional treatment options will be considered.

2- Radiation therapy

The second most frequent treatment option is the use of radiation to specifically kill cancer cells. Radiation therapy includes external beam radiation therapy and interstitial prostate brachytherapy. In interstitial prostate brachytherapy wires, catheters, seeds, or

needles sealed with radioactive substances are implanted in the prostate (Zelefsky and Whitmore, 1997; Sogani *et al.*, 1980).

3- Radical prostatectomy

The third choice is radical prostatectomy (RP) which is a surgical procedure to remove the entire prostate and, if necessary, also of nearby tissues such as seminal vesicles. Nowadays laparoscopic or robot-assisted techniques have replaced retropubic or perineal incision methods. RP is mainly recommended for patients with a low-grade and truly organ confined PCa with over a 10-year life expectancy (Xu *et al.*, 2000).

4- Hormonal therapy

In cases where the cancer has already metastasized at the time of diagnosis, where RP or radiation is not possible, or where a relapse after local treatment occurs, hormonal therapy is used. Hormonal or hormone-replacement therapy (castration) removes the male hormones or blocks their activation to stop the cancer cells' growth. It can be performed with different methods including:

- luteinizing hormone-releasing hormone analogues and antagonists that can stop the synthesis of hormones in the testicles (Zhang *et al.*, 2007)
- anti-androgens that block the hormonal action of androgens
- orchiectomy or surgical castration, which is the surgical removal of the testicles.

A systematic review and a meta-analysis of neo-adjuvant (hormone therapy given before other treatments) and adjuvant hormone therapy (hormone therapy that is given after other primary treatments) in localized or locally advanced PCa revealed a significantly increased overall survival at 5 and 10 years for adjuvant therapy following radiotherapy and a significant improvement in disease-specific survival and disease-free survival at 5 years (Kumar *et al.*, 2006).

Chemotherapy

Chemotherapy is using anti-cancer agents to kill cancer cells or stop their division irrespective of their location in the body. Chemotherapy is not considered as standard treatment for localized PCa. However, it is mainly used when the cancer does not respond to hormone therapy. Like hormonal therapy, chemotherapy will not cure PCa completely, but it can shrink the size of the primary tumor, reduce local and distant metastases, and generally slow down cancer growth, which will ideally turn PCa into a chronic disease. Furthermore, this may help to control some of the symptoms, e.g., pain or extend the patient's life. Chemotherapy (treatment with drugs, e.g., docetaxel or

paclitaxel) is really just used for the most advanced cancers when the patients' life expectancy does not greatly exceed one year.

1.2.3.5. AR and castration resistant prostate cancer (CRPC)

The male sex hormones or androgens are steroid hormones and play a key role in the development of male phenotype during fetal life and puberty. In adults they control fertility and sexual behavior. Prostate cells are androgen-dependent for growth, differentiation and maintenance. Testosterone and 5α-dihydrotestosterone are the two essential androgens involved in the development and maintenance of the prostate gland. Androgen receptor (AR) is a transcription factor of the steroid receptor family, and acts as the primary mediator of androgen action. The activation of AR signaling pathway in the prostate results in cell growth and differentiation as well as the production of prostatic fluids. The schematic illustration of AR signaling pathway is shown in Figure 1.

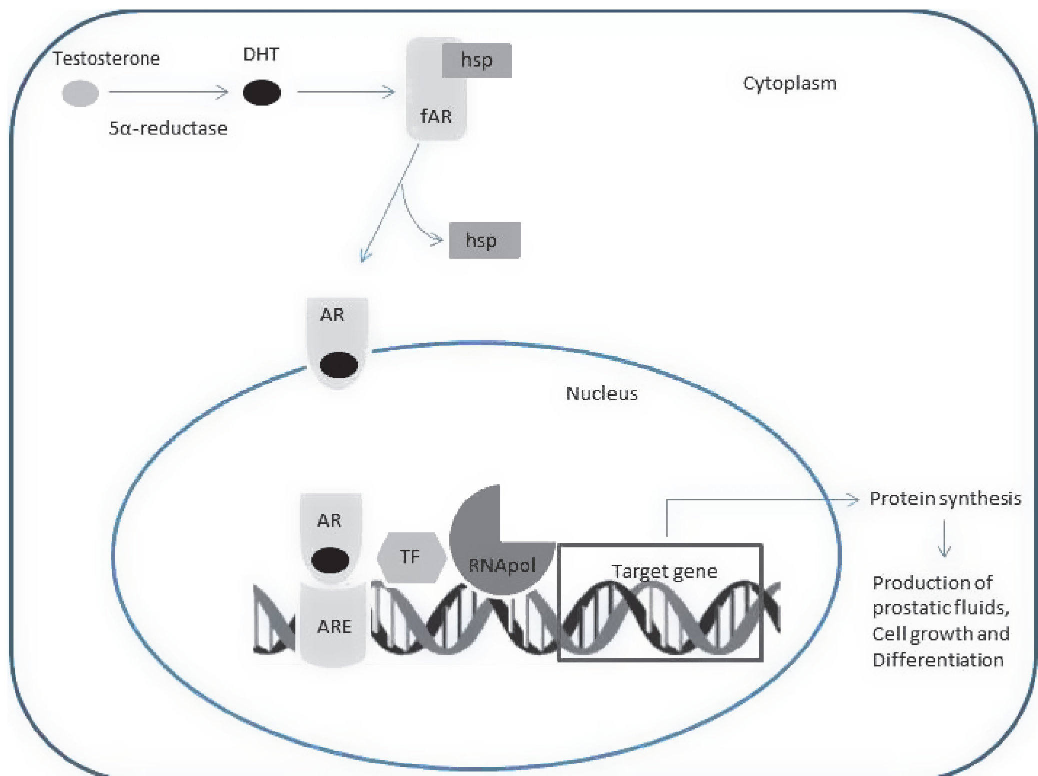


Figure 1. Schematic illustration of AR signaling pathway in prostate. DHT: 5α-dihydrotestosterone; fAR: free AR (in the absence of ligand); hsp: heat shock protein; ARE: androgen response elements; TF: transcription factors; and RNApol: RNA polymerase.

Since PCa cells are remarkably androgen dependent, all patients initially respond to androgen deprivation therapy (ADT). However, after treatment for 2–3 years, most patients will develop resistance to these traditional hormonal approaches. Some patients will respond initially to secondary hormonal manipulations before the unavoidable formation of castration-resistant prostate cancer (CRPC). Several mechanisms leading to castration-resistant behavior are known, and these enable the tumor cells to survive and grow under androgen-depleted conditions. Some of the mechanisms are listed below:

a) The bypass of the AR signaling pathway: the autocrine production by epithelial cells of some growth factors, such as FGF and IGF1 (which are normally secreted by stromal cells in response to androgens) and the activation of oncogenes and inhibition of tumor suppressor genes are additional molecular mechanisms that tumor cells may apply to bypass the AR pathway (Cheng *et al.*, 2007; Cooperberg *et al.*, 2012; Heidenreich *et al.*, 2014; Altman *et al.*, 2012), whereas the amplification and overexpression of AR have been reported in androgen-independent cancers, compared to primary tumors (Massoner *et al.*, 2013; Celis *et al.*, 2009; Pascal *et al.*, 2009; Koivisto *et al.*, 1997; McEntee *et al.*, 1996; Epstein, 1994);

b) The *de novo* synthesis of androgens or converting steroids to testosterone (Srigley *et al.*, 2000; Zoladz and Diamond, 2013; Wilkes and Tasker, 2014; Patel *et al.*, 2004);

c) AR mutation (Stamey, 2001), ligand-independent transactivation, and coactivator or corepressor alteration are further alterations of the AR pathway.

1.3. Diagnosis of PCa

Current routine diagnostic tools to investigate the presence of PCa are: the PSA test that measures the level of PSA (prostate specific antigen) in blood, digital rectal examination (DRE), and transrectal ultrasonography (TRUS). However, the definite diagnosis needs to be confirmed by the histological examination of prostate biopsy cores or specimens obtained from operations. Recently, multiparametric magnetic resonance imaging (mpMRI) has been used to distinguish between life-threatening and non-life-threatening cancers of the prostate for PCa management. It has been reported that mpMRI is a much more powerful risk-stratification tool than the PSA test (Thompson and Pokorny, 2015). PSA is a member of the kallikrein-like family of serine proteases that is almost exclusively produced by the prostate gland; a portion of that can leak to the blood stream depending on the patient's age and the condition of the prostate gland. However,

increased PSA levels are not specific to PCa and PSA may also be elevated in other, non-malignant prostate conditions, e.g., BPH and prostatitis. In 1986, the PSA blood test was approved by the US food and drug administration for the screening and follow-up of PCa patients (Stamey *et al.*, 1987). The usefulness of PSA screening in decreasing the mortality rate of PCa has been reported earlier (McShane *et al.*, 2006), but PSA test has also resulted in a large number of false positive results and over-diagnosis (McShane *et al.*, 2005). Several studies have demonstrated that the majority of men diagnosed with clinically localized PCa will not, even without treatment, develop aggressive and lethal forms of PCa (Rubio-Briones *et al.*, 2014; Brooks, 2013). For this group of patients, only active surveillance would be enough. The histological examination of biopsies is also used to confirm the clinical suspicion for PCa and it is usually performed on a sequence of 6-12 tissue biopsy cores. However, these may not truly represent the whole gland and the suspicious lesion may be missed in biopsies leading to a false negative diagnosis or under-grading and, thus, a clear diagnostic and prognostic evaluation of the cancer risk may be compromised. Therefore, new biomarkers are needed to compliment the current diagnostic tools to facilitate the early diagnosis of PCa. Most importantly, such new markers should help in discriminating between indolent and aggressive cancer phenotypes and to provide a more specific, personalized prognosis.

1.3.1. Different biomarkers for PCa

Different definitions for biomarkers have been stated. Mueller defines biomarkers as "any measurable cellular, biochemical or molecular alterations in biological media such as human tissues, cells, or fluids (Felix *et al.*, 2014)". On the basis of the definition suggested by the National Institute of Health (NIH) a biomarker is a measurable characteristic that is an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (Taylor *et al.*, 2013). This most recent definition of a biomarker covers a broader explanation and applies to any measurable cellular, molecular, morphological, or biochemical changes in biological matrices such as cells, human tissues, or fluids (Arem *et al.*, 2013; Chiriaco *et al.*, 2013). An ideal cancer biomarker should be able to distinguish between healthy individuals and patients. It should already be expressed or its expression should elevated at an early stage of the disease. It should be easy to measure, inexpensive, and useful in providing more precise predictions, and it should help to reduce mortality. Good reproducibility and the possibility to multiplex multiple markers are other characteristics of ideal cancer biomarkers.

Disease biomarkers can be divided into different types on the basis of their application. Risk markers provide early evidence of the risk of disease in people that have not been diagnosed with the disease. The earliest risk markers for cancer studies belong to

inherited genetic abnormalities, e.g., *BRCA1* and *BRCA2* mutation in breast and PCa (Cao and Sun, 2013; Feng *et al.*, 2013). Diagnostic markers such as PSA are used to identify cancer at an early stage in the general population with the objectives to identify, classify, and staging of the patients. Besides PSA, *AMACR*, *PCA3*, *TMPRSS2-ERG* and human kallikrein 2 (KLK2) are some of the well-known diagnostic marker candidates for PCa. Based on the European Association of Urology (EAU) Guidelines on PCa, DRE, serum PSA level and histopathological verification of adenocarcinoma in prostate biopsy cores or specimens from TURP are recommended as the main diagnostic tools for PCa (Heidenreich *et al.*, 2014).

PSA is a better predictor of PCa when compared to DRE or TRUS. A risk of 10–30% false negative results and missed cancers using TRUS guided biopsy has been reported by several studies (Barqawi *et al.*, 2011; Stewart *et al.*, 2001; Patel *et al.*, 2004). Already moderately increased PSA levels indicate a greater likelihood of PCa and therefore PSA has been extensively used in early detection of PCa. But using PSA test for population screening is highly controversial, because it results in excessive use of biopsies leading to overdiagnosis and overtreatment of non-aggressive cancers with serious adverse effects. It was recently reported by the European Randomised Study of Screening for Prostate Cancer that screening of PCa using PSA does result in a substantial reduction in prostate cancer mortality (Schroder *et al.*, 2014). Due to excessive use of biopsies, with resulting high rate of overdetection and overtreatment, PSA does not support initiation of wide population-based screening. However, the current EAU guidelines for PCa recommend that early PSA testing should be offered to those men who are at elevated risk for PCa (e.g. men over 50 years of age, men over 45 years of age with a family history of PCa, and to African-Americans) (Heidenreich *et al.*, 2014).

Risk of PCa in men with PSA level between 0-4 ng/mL varies from 6.6 to 27%. PSA values between 4.0 and 10.0 ng/ml are called the "gray zone" and a positive predictive value of only 26% has been reported for PSA in this range (Catalona *et al.*, 1994). This results in unnecessary biopsies, discomfort, anxiety, and the risk of infection for the remaining 75% of men. To improve the sensitivity and specificity of the PSA test different modifications of PSA test, such as PSA density (level of serum PSA/prostate volume), PSA velocity (absolute annual increase in serum PSA ng/mL/year), PSA doubling time (the exponential increase in serum PSA over time), free/total PSA ratio and Prostate Health Index (PHI) have been suggested. The use of a panel of kallikrein markers (total, free, and intact PSA, and human kallikrein 2 (KLK2)) to reduce the rate of unnecessary biopsies has been reported (Vickers *et al.*, 2008). This concept, which is now commercially available as the 4Kscore® Test (OPKO Diagnostics, LLC), uses the previously mentioned panel of four kallikreins together with clinical information in an algorithm to calculate an individual's risk for aggressive prostate cancer on prostate

biopsy. It has been shown that the 4Kscore test, as a follow-up test after abnormal PSA and/or DRE test results, can improve the specificity for predicting the risk of aggressive prostate cancer as well as reduce unnecessary prostate biopsies (Punnen, Pavan and Parekh, 2015; Konety *et al.*, 2015).

The Progensa PCA3 assay (Groskopf *et al.*, 2006), a urine test that measures *PCA3* mRNA in post-DRE urine sediments and has been reported to be superior to total PSA and F/T PSA percentage for detection of PCa, is another test that is recommended by the EAU guidelines on PCa (Heidenreich *et al.*, 2014). Recently several other tests such as Oncotype DX® (provides Genomic Prostate Score (GPS) and aggressiveness of PCa) (Klein *et al.*, 2012), Polaris® (measures the aggressiveness of PCa) (Cuzick *et al.*, 2011; Cooperberg *et al.*, 2013), and Confirm MDx (addresses false-negative biopsy) (Stewart *et al.*, 2013) have been developed to improve diagnosis and prognosis of PCa, but they are not recommended by EAU guidelines yet.

AMACR is routinely used as a tissue biomarker to support the diagnosis of PCa. It is also useful for detection of small carcinoma foci in needle biopsies when combined with a basal cell marker such as keratin 5/6 or p63 (Jiang *et al.*, 2002b; Jiang *et al.*, 2002a; Kristiansen, 2009). *TMPRSS2-ERG* fusion detection in urine samples has resulted in 90% specificity and 94% positive predictive value for PCa detection (Hessels *et al.*, 2007). The high specificity of the *TMPRSS2-ERG* test makes it a potential candidate to be served in the clinic as a viable biomarker for investigating the presence or absence of PCa (Perner *et al.*, 2007).

In contrast, prognostic markers are most useful for patients who already have been diagnosed with cancer. They are used to determine the risk of cancer progression, its progression towards increased aggressiveness, poor patient survival, and patterns of cancer recurrence. E-cadherin, *MMP9*, and *VEGF* are examples for PCa prognosis markers (Lee *et al.*, 2013), although none of them are utilized in clinical practice yet. Predictive markers are particularly important because of their role in estimating the likelihood of specific treatment outcomes (due to the fact that response to treatment correlates with the presence or absence of that particular biomarker). In some cases, predictive markers may also be functionally involved in cancer progression towards more aggressive behavior, failure of therapy, and resistance to chemotherapy. For example, *BCL-2* expression could be used as a biomarker to identify those PCa patients who may respond (or fail to respond) to taxane-based chemotherapy (Ibrayev *et al.*, 2013).

1.3.2. Types of biological samples available for identification of PCa biomarkers

Blood, urine, semen, and prostate tissue are the most frequently used and clinically available biological specimens that can be used in PCa biomarker identification. Blood

samples contain various amounts of human proteins that are diagnostically relevant, exosomes, and circulating cancer cells (CTC). All of these can be used for diagnoses that are based on proteins, DNA, or RNA. Urine samples, like blood, also contain exosomes and exfoliated cells. Due to the non-invasive nature of urine sampling, these samples have become particularly popular in protein, DNA, and RNA-based marker research. PCa biomarkers can be divided into different groups: protein-based, DNA-based, lipid/ metabolites-based and RNA-based markers. Proteomics techniques (e.g., mass spectrometry, electrophoresis, chromatography, and enzyme-linked immunosorbent assays (ELISA)) are most routinely used to identify protein-based biomarkers and to validate their usefulness, while genomics techniques (e.g., oligonucleotide microarray, RT-PCR, and next-generation sequencing) are used for the identification and validation of DNA and RNA-based biomarkers.

1.3.3. Protein-based biomarkers

Among the protein markers used for detecting PCa, PSA is the first fully accepted and approved biomarker that has been commercially used in clinics since the early 1990s. Furthermore, prostatic acid phosphatase (PAP), alpha-methyl Co-A racemase (AMACR), prostate-specific membrane antigen (PSMA), prostate stem cell antigen (PSCA), early PCa antigen (EPCA), and human kallikrein 2 (KLK2) are examples of additional, protein-based biomarkers that are used in PCa research.

1.3.4. DNA-based biomarkers

Genetic alterations: Several hereditary and sporadic genomic alterations (including mutations, chromosomal arrangements, gene deletion, gene amplification, etc.) have been reported in PCa. Table 3 shows a list of known gene alterations in PCa.

Table 3. Examples of DNA-based PCa biomarkers

Gene	Function	Alteration in PCa
CHEK2	Involved in DNA damage signaling pathway, upstream of P53	Deletion
NBN	Involved in cell cycle check point and DNA double-strand break repair	Deletion
NKX3-1	Tumor suppressor gene	Deletion
CHD1	Adhesion molecule	Deletion
PTEN	Tumor suppressor gene	Mutation
RB1	Tumor suppressor gene	Mutation
MYC	Transcriptional activator	Amplification
EGFR	Growth factor	Amplification
AR	Androgen receptor	Deletion, Amplification

Epigenetic alterations: It has been reported that epigenetic changes occur already in the early stages of PCa, and at a higher frequency than genetic changes (Gaster, 2013). Histone modification and altered methylation are the two main epigenetic alterations that have been reported for PCa. DNA methylation is catalyzed by DNA methyltransferase enzyme that includes the addition of a methyl group to the 5′-carbon of cytosine in CpG sequences. The majority of CpGs in the human genome are methylated, whereas most of the CpG islands (small clusters of CpGs that are found in the 5′region close to the promoter of the genes) remain unmethylated which contributes to these regions being transcriptionally active (facilitating the binding of transcription factors and co-factors). Alteration in DNA methylation is one of the earliest events in tumorigenesis. DNA methylation in promoter regions represses the transcription of these genes, and it is also considered to be a defense mechanism against mobile genetic elements. One of the most common and well-studied epigenetic alterations in PCa is the hypermethylation of tumor suppressor genes and genes involved in other pathways. Hypomethylation (global and gene-specific) has also been shown to be involved in human PCa progression. A list of the genes affected by altered DNA methylation in PCa is shown in Table 4.

Table 4. Examples of epigenetic alterations in PCa

Gene symbol	**Pathway**	**Epigenetic alteration**
CDKN2A, CCND2	Cell cycle control	DNA hypermethylation
GSTP1, MGMT	DNA damage repair	
RARβ,ENDRB, CDKN1C, TIMP2	Tumor suppression	
AR, ESR1, ESR2, ERα, ERβ, RARRES1	Hormone regulation	
uPA, HPSE	Tumor invasion and metastasis	DNA hypomethylation
CAGE	Cellular proliferation	

GSTP1 gene encodes an enzyme which is involved in detoxification and protection of DNA from oxidants and carcinogens. Hypermethylation of *GSTP1* has been reported in more than 90% of PCa and about 70% of PIN lesions (Nakayama *et al.*, 2004). It has been reported that detection of *GSTP1* methylation in urine sediments collected after prostatic massage results in 75% sensitivity and 98% specificity for PCa detection (Woodson *et al.*, 2008). In that study, *GSTP1* methylation was more frequently detected in urine samples of patients with a higher tumor volume and later stage disease. Detection of *GSTP1* methylation in 20% of men with prediagnostic PSA less than 4 ng/ml and in 90% of the men with prediagnostic PSA more than 4 ng/ml PSA has also been reported (Woodson *et al.*, 2008). Histone modifications including methylation, phosphorylation, acetylation, and deacetylation are other types of epigenetic alterations that regulate gene expression in PCa. *CAR, CPA3*, and *RARB* are some examples of

genes, which are suppressed or downregulated in PCa by histone deacetylation (Roobol *et al.*, 2013; Belbase *et al.*, 2013; Miocinovic *et al.*, 2013).

Histone methylation is an emerging area of research particularly in PCa, but it cannot be outlined in great detail here. Nevertheless, some epigenetic modifiers, e.g, LSD1 and EZH2 have been specifically associated with the progression of PCa and development of CRPC. For example, the Histone demethylase LSD1 (KDM1A) is also thought to play a role in prostate and other cancers, as poor patient outcome has been correlated with an increased expression of this gene. Therefore, the inhibition of KDM1A and, potentially, of other epigenetic factors has attracted much attention as a possible treatment for cancer.

1.3.5. RNA-based biomarkers

The development of new transcriptome technologies, such as microarrays and next-generation sequencing, have resulted in growing interest in the field of RNA biomarkers. RNA-based biomarkers are also interesting due to the fact that they reflect the functional state and the state of the biological systems of the cells. Moreover, RNA expression levels integrate both the genetic and epigenetic regulation mechanisms of these genes. Among different kinds of RNAs, micro RNA (miRNA), long non-coding RNA (ln-RNA), and mRNA are currently extensively studied in PCa.

1.3.5.1. miRNAs in PCa

miRNAs are short (approximately 17-25 nucleotides long) non-coding RNAs that form 1- 3 percent of the mammalian genome (Vasarainen *et al.*, 2013). miRNAs play a key role in the regulation of gene expression at the post-transcriptional level. They perform this role by either translation repression or mRNA degradation (Carter, 2013; Baum, 2013; Gulati and Etzioni, 2013). It has been reported that some of the miRNA function essentially like tumor suppressors (i.e., suppressing the activity of oncogenic genes and proteins), while others have oncogene-like action (Kobayashi *et al.*, 2013; Zuzana *et al.*, 2013; Altarac, 2013; 2013; Simmons, 2013; Salomon, 2013; De Coninck, Braeckman and Michielsen, 2013). Furthermore, it has been shown that the expression levels of most miRNAs are tissue-specific (Makovey, Stephenson and Haywood, 2013). The expression of miRNAs is frequently altered during cancer development and metastasis, and miRNAs, therefore, can be used as diagnostic or prognostic markers. The stability of miRNAs in the blood stream makes circulating miRNAs a useful target, particularly suitable for less invasive biomarker studies based on blood or urine (Xiao *et al.*, 2014; Lee and Jung, 2013). Different molecular technologies (e.g., microarrays, quantitative RT-PCR, and deep sequencing) have been used for miRNA profiling. Several studies have discovered different miRNAs in association with PCa as diagnosis markers (miRNA-107, miRNA 141, and miRNA93) (Kilpelainen *et al.*, 2013; Melnikow *et al.*,

2013; Howard, 2013), prognosis (miRNA-200b, miRNA-375 and miRNA451) (Sammon, Sukumar and Trinh, 2013; Melnikow *et al.*, 2013) and predictive markers (miRNA-21) (Lippi, Mattiuzzi and Cervellin, 2013).

1.3.5.2. Long non-coding RNAs in PCa

Another group of non-coding RNAs are long non-coding RNAs. Their length varies from 200 bases up to 100kb. lnRNAs have much lower expression level in comparison with mRNA and they do not encode any protein but instead they have regulatory feature (Wang *et al.*, 2013). lnRNAs are divided into two different groups on the basis of their genomic localization: intergenic lnRNAs and intronic lnRNAs. *PCA3* is the most well-known and clinically relevant lnRNA-based biomarker for PCa. An overexpression of *PCA3* has been reported in over 95% of PCa cases (Howard *et al.*, 2013). Urine-based PCA3 tests are now commercially available and have been reported to provide higher specificity in comparison to PSA (Shaw, Scott and Ferrante, 2013; Howard *et al.*, 2013). In one of the most promising studies in this field, conducted by Prensner *et al.*, the authors found 121 new PCa-associated lnRNAs (Ilic *et al.*, 2013). Most interestingly, PCa-associated non-coding RNA transcripts-1 (*PCAT-1*) showed very significant overexpression in high-grade organ-confined and metastasized PCa. Furthermore, PCa non-coding RNA-1 (*PRNCR1*) has been reported to be upregulated in aggressive PCa (Chowdhury *et al.*, 2013).

1.3.5.3. mRNA transcripts as PCa biomarkers

As different cellular pathways (e.g., differentiation, survival, etc.) are reflected by altered gene expression patterns, mRNA transcripts are suitable targets for therapeutic and diagnostic cancer research. Several techniques have been developed for gene expression studies, e.g., northern blotting, RNase protection assay, *in situ* hybridization, RT-PCR combined with DNA sequencing, and microarrays (Parker and Barnes, 1999; Hod, 1992; Saccomanno *et al.*, 1992; Weis *et al.*, 1992; Bucher, 1999). During the last few years, dramatic improvements in the available methods and the development of new high-tech methods, such as microarray and next-generation sequencing, have made the whole-genome expression studies possible. This has had a fundamental impact on different fields of cancer research, e.g., molecular diagnostics, tumor development and progression pathway classification, novel drug target identification, as well as drug discovery and development. Microarray-based gene expression profiling, quantitative RT-PCR and, more recently, novel DNA and RNA sequencing methods are now commonly used to identify novel biomarkers in cancer diagnostics and a brief summary of them is given below.

1.4. Microarray and gene expression profiling

The microarray technique was developed in parallel by Lipshutz *et al*. (Lipshutz *et al.*, 1999), and Schena in the laboratory of Patrick Brown (Schena, 1995). DNA microarrays provide the unique possibility to study and monitor the expression levels of thousands of genes in a quantitative, rapid and reproducible manner. Spotted array and high-density oligonucleotide array are still the two most widely available basic types of array technologies. The microarray technology is now rapidly being replaced by next-generation DNA sequencing technologies, although both technologies are likely to co-exist for some time to come.

Spotted microarrays were initially manufactured by using xyz robots to spot pre-synthesized oligonucleotides (PCR products, cDNA) on specially coated glass slides (Schena *et al.*, 1995). Sequences to be printed are chosen from well-characterized genes and public databases of expressed sequence tag (EST). About 80,000 spots can be fitted onto the surface of a slide. The possibility of concurrent analysis of two different samples provides the advantage of testing a control sample that is related to the experimental sample. This could also be considered as a disadvantage for this technology because it only provides a relative estimation between two samples (Macgregor and Squire, 2002).

Oligonucleotide arrays were pioneered by the US-based company Affymetrix. Photolithography is used to generate thousands of *in situ* synthesized oligomers onto glass slides called GeneChips^{TM} (Fodor *et al.*, 1991). In the latest versions of Affymetrix arrays, 10-20 different oligonucleotide probes for each gene are specifically designed from the 3′ end of the transcript. Approximately 500,000 probes can be deposited onto a single GeneChip^{TM} and over 40,000 genes and ESTs can be analyzed by one chip. The ability to measure the absolute gene expression levels in many samples is the main advantage of this technique (Macgregor and Squire, 2002), and it also provides the subsequent bioinformatics analysis with assay speed and standardized methodology. Since the early 2000s, microarray-based gene expression profiling has been used to measure the expression of thousands of genes in parallel and across many different biological samples, allowing researchers to compare their expression levels with each other. Numerous larger-scale microarray studies have used gene expression profiling to identify novel genes that could have potential as PCa diagnostic tools and prognosis markers (Dhanasekaran *et al.*, 2001; Magee *et al.*, 2001; Welsh *et al.*, 2001; Luo *et al.*, 2001; Stamey *et al.*, 2001; Luo *et al.*, 2002a; Singh *et al.*, 2002; Rhodes *et al.*, 2002; Varambally *et al.*, 2002; Henshall *et al.*, 2003; Glinsky *et al.*, 2003) as well as predictive markers (Latil *et al.*, 2003; Glinsky *et al.*, 2004; Schlicht *et al.*, 2004; Li *et al.*, 2005; Sooriakumaran *et al.*, 2009). Since the development of fast DNA sequencing methods (since 2010), the use of microarrays has been gradually declining, but the vast amount

of data generated and deposited in open access, public data repositories along with the published reports will continue to provide a very useful base for biomarker identification.

1.5. DNA and RNA sequencing

The initial sequencing and analysis of the human genome by using conventional Sanger sequencing technology took about 13 years (de Jong *et al.*, 2001). In contrast, the next-generation sequencing (NGS) technologies which have revolutionized the genomic research have reduced the required time to a single day. Nowadays, a panel of different NGS technologies, based on different technology platforms, have been developed for the parallel sequencing of millions of small fragments of DNA (“deep sequencing”). The term second-generation sequencing is used to describe an older PCR-based sequencing method in contrast to the amplification-free, third-generation techniques that can sequence a single molecule (Schadt, Turner and Kasarskis, 2011). The application of these advanced sequencing technologies makes it possible to discover genetic and epigenetic changes possibly responsible for functioning in the development and progression of cancer. In comparison to microarray technologies, RNA/DNA sequencing by NGS methods is cheaper and provides enhanced resolution, higher dynamic range, and better reproducibility. NGS methods also make it possible to identify mutations and novel transcriptomes, such as non-coding RNAs, splice variants, and fusion genes, which were not detectable with microarray technology.

1.6. Real-time PCR methods

The principles for polymerase chain reaction (PCR) were developed by Karry Mullis (Mullis *et al.*, 1986). PCR allows the exponential amplification of any DNA molecules of suitable length. In quantitative PCR, this is further combined with the incorporation of fluorescent dyes or the use of fluorescently labeled DNA probes. The detected amount of fluorescence correlates with the original number of DNA sequences which serve as templates for DNA amplification. The kinetics of DNA amplification and the accumulation of amplified target gene fragments allow the quantitative determination of specific gene products in real-time. Coupling the reverse transcription technique with subsequent PCR enables the amplification and detection of RNA molecules (Rappolee *et al.*, 1988). Several advantages, e.g., a homogenous assay avoiding post-amplification processing, a wider dynamic range, and quantification possibility, have made RT-PCR one of the most widely used methods in gene expression studies. Real-time PCR is a relatively complex assay and numerous factors can affect the sensitivity, specificity, and reproducibility of the assay. Some of these factors are discussed below.

1.6.1. Different quantification strategies

The quantification of RT-PCR data can be based on two different principles: relative and absolute quantification. In relative quantification, the target gene expression alteration is calculated in relation to a group of untreated control samples or samples from different time courses. In absolute quantification, the absolute copy number of the transcript is calculated on the basisi of a dilution series of known concentrations in which the target gene is used as a reference to generate a quantitative standard curve. These standard curves rely on a linear relationship between the threshold cycle of an unknown sample and the initial amount of RNA or cDNA in the sample. By comparing the DNA amplification in the experimental sample to the standard curve, the concentration of DNA in the sample can be precisely calculated.

1.6.2. Normalization of RT-PCR

Normalization is very important in RT-PCR in adjusting sample-to-sample variation and evaluating the presence of any inhibitors in the reaction mixture; it is used as a means to avoid or exclude false negative results. Different strategies can be used to minimize and correct these errors. The total RNA concentration can be used to normalize the measured expression level of the target gene, but this method suffers from several limitations: First, the level of RNA can be affected by different cellular processes. Secondly, extraction and reverse transcription efficiency and the quality of RNA may seriously affect the final outcome. A frequently used approach to avoid these limitations is to normalize the RT-PCR results against a stably expressed control gene that could also be used as a positive control. Housekeeping genes (e.g. *GAPDH* and *β-actin*) are generally assumed to show stable and constant expression levels across all of the cells or tissues examined, and have been widely used as controls in gene expression assays. However, frequent observations of significant alterations in the expression of housekeeping genes in various stages of different diseases have raised serious questions about the reliability of this method (Li and Shen, 2013). Few studies have attempted to investigate the application of tumor specific genes for normalization of gene expression measurements in tumor tissues. *HPRT* has been reported as a suitable gene for normalization purposes for cancer research (de Kok *et al.*, 2005). Another reliable alternative approach is to add, already during the RNA extraction, a known amount of artificially synthesized RNA as a reference gene. The spiking of RNA samples with a known concentration of an artificial RNA can be used to normalize the errors that occur during extraction and reverse transcription (Nurmi, Lilja and Ylikoski, 2000a; Huggett *et al.*, 2005). However, this approach does not provide normalization for changes taking place in RNA samples before extraction, e.g., during sample collection and storage. But, it still represents a more reliable approach for the absolute quantification of RNA expression.

1.6.3. Detection chemistries

Nowadays, there are several different techniques for the detection of amplified products. Fluorescent dyes have been widely used in different detection techniques. Using fluorescent dyes (e.g., SYBR Green) that bind specifically to double-stranded DNA (dsDNA) is the simplest method (Morrison, Weis and Wittwer, 1998), and it is the most widely used. The intensity of fluorescence increases proportionally to the dsDNA concentration. The disadvantages of this method are that the method does not allow multiplexing (the co-amplification and distinction of several DNA molecules in the same reaction), and it produces a high rate of false positive results due to the unspecific binding of fluorescent dyes to any dsDNA (Wittwer *et al.*, 1997a; Simpson *et al.*, 2000). Hybridization probe (Wittwer *et al.*, 1997b), hydrolysis probe (Gibson, Heid and Williams, 1996; Clegg, 1992), molecular beacons (Tyagi and Kramer, 1996), and scorpions (Whitcombe *et al.*, 1999) are other detection chemistries that have been used in RT-PCR. They all rely on the principle of fluorescence resonance energy transfer (FRET). Conventional short-lifetime fluorophores are used to label the probes. In some of these techniques, a "dark" non-fluorescent chromophore called a quencher is used to scatter the received energy from the fluorophore as heat, when it is in a close proximity to it. Although different methods, e.g., quencher dye, have been applied to reduce the background problem, high background and low signal-to-background ratio still remain major limitations in all of the previously mentioned detection techniques. Therefore, the autofluorescence which is inherent to all biological molecules and plastic matrices, combined with the high background of short-lived fluorophores may affect the sensitivity and reproducibility of the quantification of low-abundance targets in particular (Karrer *et al.*, 1995). The application of probes bearing a label of long-lifetime luminescent lanthanide chelates, combined with time-resolved fluorometry (TRF) is an extremely suitable approach to overcome the high background observed with short-lifetime fluorophores (Lövgren, 1990).

2. AIMS OF THE STUDY

The general purpose of this study was the identification, characterization, and clinical validation of novel biomarkers for diagnosis and prognosis of PCa by using gene expression.

The specific aims included:

I. To develop and optimize a highly sensitive reporting chemistry to be used for the development of truly quantitative and internally standardized qRT-PCR assays, and the comparison of CTCs in blood samples with those in prostate tissue samples to find a suitable sample matrix for our gene expression studies.

II. To examine the diagnostic potency of *AMACR* and *AR* mRNA expression levels in PCa detection with novel qRT-PCR assays. To investigate whether the the candidate biomarkers are present and informative in histologically benign tissues of the cancerous prostates.

III. The identification and validation of novel biomarkers for PCa, using bioinformatics data mining of large-scale, public gene expression datasets, and clinical biomarker validation by qRT-PCR; and the functional evaluation of candidate genes using siRNA technique in 2D and 3D culture models.

IV. To evaluate the diagnostic power of the 11 RNA biomarkers (*ACSM1, AMACR, CACNA1D, DLX1, PCA3, PLA2G7, RHOU, SPINK1, SPON2*, *TMPRSS2-ERG,* and *TDRD1*) in biopsy cores obtained from apparently benign areas targeted by magnetic resonance imaging (MRI) from men with a clinical suspicion of PCa.

3. SUMMARY OF MATERIALS AND METHODS

A more detailed description of the materials and methods used in this study is available in the original publications (I-IV).

3.1. Clinical samples

Six different sample cohorts were used in this study. A written informed consent was obtained from all participants. The study protocol was approved by the local Ethics committee and it was in accordance with the Helsinki Declaration of 1975 that was revised in 1996. The summary of tissue and blood sample cohorts used in this study is presented in Table 5 and more details about each cohort are represented in chapters 3.1.1 - 3.1.6.

Table 5. Summary of clinical samples used in this doctoral study.

Collection institute	Study population	Sample matrix	Number of samples	Publication
TYKS	Men with PCa	Blood	12	I
DBUT	Healthy male and female	Blood	5	I
TYKS	Men with localized PCa	Tissue from RP specimens	138	I, II
TYKS	Men with localized PCa	Tissue from RP specimens	178	III
TYKS	Men with localized PCa	Cross sections from RP specimens	3	III
SUH	Men with bladder cancer	Tissue from CP specimens	19	II, III
TYKS	Men with clinical suspicion of PCa	MRI-targeted biopsy	99	IV

TYKS: Turku University Hospital, Turku, Finland; DBUT: Division of Biotechnology, University of Turku, Turku, Finland; SUH: Skåne University Hospital, Malmö, Sweden.

3.1.1. Prostate tissues from patients with PCa (Cohort A) (I,II)

Prostate tissue samples were collected from 79 patients operated by radical prostatectomy (RP) because of clinically localized PCa at Turku University Hospital, Turku, Finland (TYKS). From each fresh prostatectomy specimen two small samples were taken immediately after surgery, one from the suspected cancerous area, the other from an adjacent, suspected benign area. Half of each tissue sample was sent to experienced pathologists at TYKS for histopathological examination, while the other half was stored in guanidine isothiocyanate buffer (GITC buffer, containing 4 mol/L

guanidine thiocyanate, 25 mmol/L sodium citrate dehydrate, 0.5% W/V sodium lauryl sarcosinate, and 0.7% V/V 2-mercaptoethanol) and stored at -80 ˚C until further processing. On the basis of histopathological examination results for some patients both samples had been taken from either benign or cancerous areas. For 59 prostates both samples were examined; in 9 and 11 cases (cancerous or benign, respectively), only one sample per prostate was examined. In total, 69 RP-Be samples and 69 RP-PCa samples were examined.

3.1.2. Prostate tissues from patients with PCa (Cohort B) (III)

Due to running out of cDNA from first RP tissue sample cohort, another tissue sample cohort was processed. 180 prostate tissue samples were collected from 90 PCa patients, operated by radical prostatectomy (RP) in (TYKS) between 2004 and 2007. The samples were collected, examined, and stored as described for cohort A. Because of a technical problem during RNA extraction, two samples were excluded, which resulted in 178 samples (104 RP-Be samples and 74 RP-PCa samples) for further analysis.

3.1.3. Prostate tissue from cross-section of PCa patients (II)

Single cross-sections of the prostate were collected from three men with PCa who had undergone RP at TYKS. A 2 mm horizontal mid-plane tissue slice covering the entire gland was obtained from each prostate. Prostate slices were fixed on a Styrofoam plate with grid guide on it and cut into 5x5 mm pieces. This resulted in 44-62 pieces depending on the size of the prostate. The samples were stored in RNAlater (Qiagen, Germany) at -20 ˚C until RNA extraction. A tissue section from adjacent inferior and superior sides of the slice used for mRNA measurements was fixed in formalin and embedded in macro paraffin blocks (FFPE) for histological examination. The histology of the tissue samples collected for mRNA experiments was determined by matching each sample with the corresponding area on sections used for histological examination. For immunohistochemistry experiments macrosections of 5 µm in thickness were cut from the FFPE blocks of each prostate, next to the HE-stained sections.

3.1.4. Prostate tissues from men with bladder cancer (II, III)

Prostate tissue samples were collected from 19 men who had bladder cancer, and who underwent cyctoprostatectomy at Skåne University Hospital in Malmö, Sweden. One tissue sample was collected from each specimen and samples were stored fresh frozen at -80 ˚C until RNA extraction. The specimens were further examined histologically by a pathologist. The results revealed that seven glands were tumor-free, while the other 12 contained an incidental tumor but the mRNA experiment samples had been collected from a tumor-free area.

3.1.5. Prostate tissue biopsies from men with clinical suspicion of PCa (IV)

Prostate tissue biopsies were collected from 100 men with a clinical suspicion of PCa (PSA 2.5-20 ng/ml and/or abnormal DRE). Patients underwent magnetic resonance imaging (MRI) at 3 teslas before biopsy. A total number of 6+6 biopsies were systematically collected from each patient. In the case of a suspected lesion in the MRI, two cognitively targeted biopsies were collected from the index lesions. Finally, two fresh biopsies were obtained for biomarker research. These biopsies were obtained from the peripheral zone of the prostate without clinical or radiological evidence of tumor. Tissue sample cores were placed in RNA-stabilizing solution (RNAlater, Ambion, Austin, TX, USA) and stored at -80 °C until RNA extraction.

3.1.6. Blood samples from PCa patients and healthy individuals (I)

Blood samples were taken from 12 prostate cancer (PCa) patients with metastasized PCa at TYKS, and from 3 healthy males and 2 healthy females at the University of Turku Division of Biotechnology. From each individual, 2.5 mL of blood were collected in PAXgene blood RNA tubes (PreAnalytix, Germany) and stored at -20 °C until further processing.

3.2. RNA extraction and reverse transcription

PAXgene Blood RNA kit (PreAnalytix, Germany) was used for the extraction of total RNA from blood samples. According to the manufacturer's instructions, the samples were first incubated for 24 h at room temperature. For the extraction of total RNA from tissue samples, RNeasy mini kit (Qiagen, Germany) was used according to manufacturer's instructions. A known and fixed amount of artificial mmPSA mRNA (*in vitro* mutated form of PSA) (Nurmi *et al.*, 2000a) was added as internal standard during the extraction procedure after cell lysis. To degrade and remove the genomic DNA contaminants an additional DNase I (Qiagen) treatment step was performed according to manufacturer's instructions.

The quality of the extracted RNA was characterized by gel electrophoresis and the RNA concentration was measured using Nanodrop (Thermo, USA) spectrophotometer. The extracted RNA was reverse-transcribed to cDNA by using High Capacity cDNA Archive Kit (Applied BiosystemsUSA) following the manufacturer's instructions. The cDNAs were stored at -20 °C.

3.3. Internal and external RNA standards (I, II, III, IV)

A fixed and known amount (10^6 molecules to each tissue or blood sample) of a synthetic mutant form of wild-type *KLK3*gene (which encodes for PSA protein) RNA was used as internal standard to facilitate monitoring the loss of RNA during extraction and reverse transcription processes. The mutant mmPSA form contains, in comparison to the wild-type, an inversion and deletion. A control representing the maximum yield of internal standard RNA was prepared by the reverse transcription of the same amount of standard RNA as was added to each sample.

For each mRNA target a dilution series of purified PCR products was used as a template for the construction of a standard curve for the quantitative measurements. End-point PCR assays and gel electrophoresis were used to produce and detect the target gene. The PCR products and the target DNA were purified from agarose gel. The purified PCR product concentration was measured by using Picogreen reagent kit (Invitrogen, USA) and was diluted to different concentrations.

3.4. Real-time PCR

Two different real-time detection principles of PCR amplification products were used in this study. The applicability of a new detection method for PCR amplification products, called lanthanide chelate complementation (LCC) was compared with a previously published detection method based on hydrolysis enhanced luminescent chelate (HELC) chemistry. In both methods lanthanide chelates were used for probe labeling, and time-resolved fluorometry (TRF) was used for the detection of fluorescence emission (Figure 2).

To investigate the applicability of the new LCC reporting chemistry for real-time qRT–PCR and to compare its performance with HELC chemistry as a reference method, two different assays were developed for *KLK3* gene.

Specific real-time PCR assays based on HELC detection chemistry were designed and developed for target genes including *KLK3*, *AMACR, AR, ACSM1, CACNA1D, TDRD1, RHOU, LMNB1, DLX1, PLA2G7*, and *SPON2*. Previously developed and described real-time PCR assays utilizing HELC detection chemistry (Vaananen *et al.*, 2015; Vaananen *et al.*, 2013) were used for *PCA3, SPINK1, TMPRSS2-ERG III*, and *TMPRSS2-ERG IV* genes.

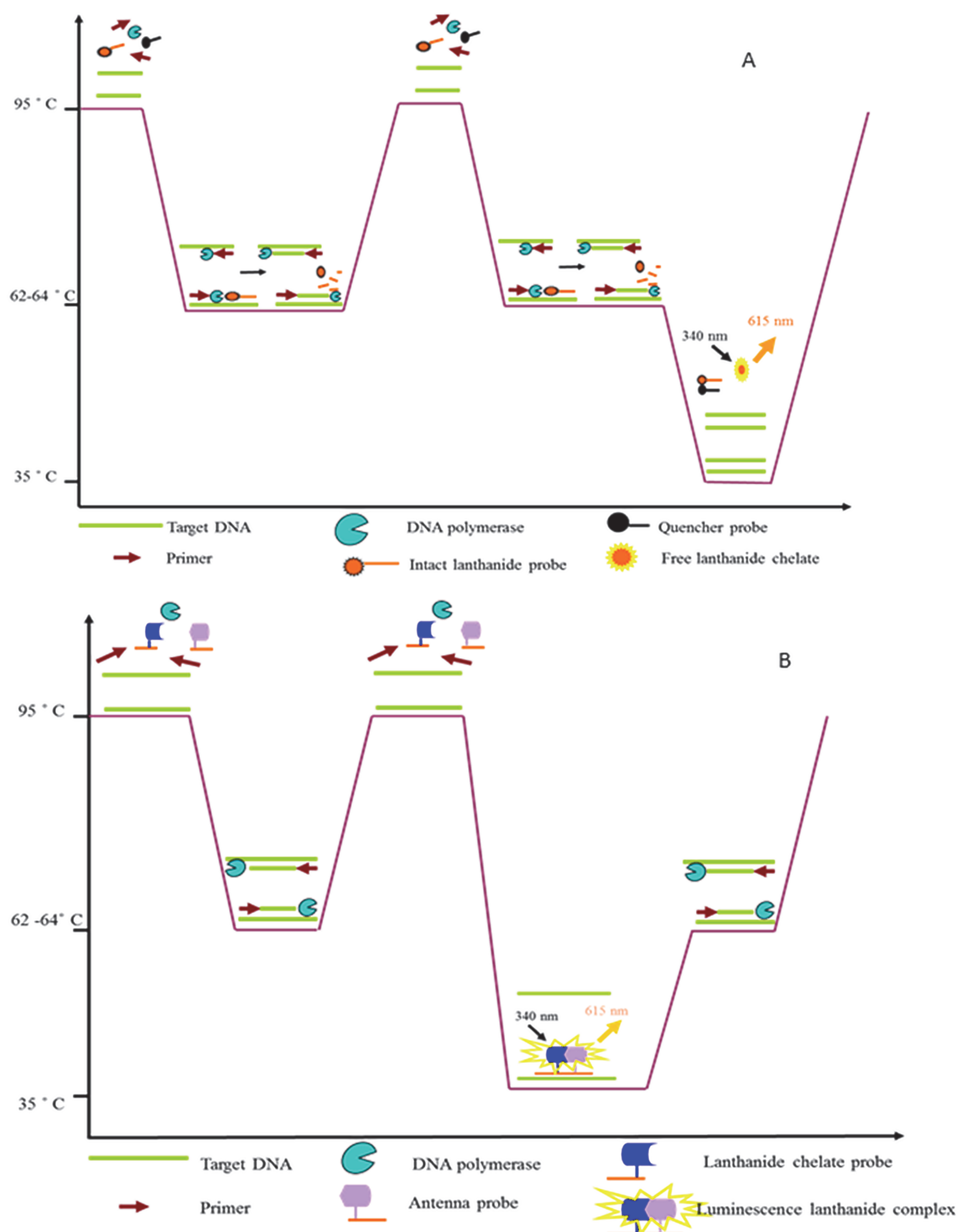


Figure 2. Schematic illustration of both methods for real-time detection of PCR amplification. (A) Assay principle of the HELC method, (B) Assay principle of LCC method. (Figure from original publication I.)

3.4.1. Reagents and temperature profile conditions

Oligonucleotide primers and probe sequences are shown in Table 6. Quencher probes were purchased from Thermo (USA) and labeled with either QSY-7 or Dabcyl at the 3’

end. Reporter probes with the amino linker C6 modification at the 5′ end of HELC probes, lanthanide carriers for LCC probes, and LCC antenna probes with a C2 amino-modification at the 3′ end (enabling labeling) were also ordered from Thermo (USA). Reporter probe labeling was performed in-house following a previously described procedure (Nurmi *et al.*, 2000b). Another previously reported procedure was also applied to the labeling of the lanthanide carrier and antenna probe for the LCC probes (Karhunen *et al.*, 2010).

Each PCR was performed in a total volume of 25 µl containing 2.5 µl of cDNA template or DNA standard. Instead of DNA template, 2.5 µl of DMPC-treated H_2O were used for negative control PCR reactions. PCR reactions were performed on a 96-well plate (Hard-shell PCR plate BLK/WHT, Bio-Rad or ABgene Thermo-Fast 96 Robotic plate white).

PCR amplifications were performed in a PTC-200 MJ Research Thermal Cycler (MJ Research, USA). A thermal profile consisting of a 15 s denaturation at 95 ˚C, followed by 1 min of annealing and extension at 64 ˚C was used for both HELC and LCC probes. A higher annealing and extension temperature was used during the first 10 cycles of amplification to improve the specificity of the assays. The annealing and extension temperature was decreased to 62 ˚C after 10 cycles, and the procedure was continued for 5 more cycles. Further amplification followed, with 30 cycles of 15 s denaturation at 95 ˚C, and annealing and elongation for 1 min at 62 ˚C. The TRF measurement was performed for the HELC method at the end of every other annealing/elongation step, starting from cycle 15. To avoid having an extra amplification step due to raising the temperature from the measurement point to the denaturation point, TRF measurement was carried out in the LCC probe-based assay at the end of every other denaturation step, starting from cycle 15. The measurement temperature for both methods was 35 ˚C; 15 s after achieving this temperature, the thermal cycler was paused and fluorescence signals of all reactions were recorded by a fluorometer (Victor 1420 multilabel counter, PerkinElmer Life Sciences, Finland). Additional 10-minute incubation at 95 ˚C was used at the beginning for enzyme activation when AmpliTaq Gold DNA polymerase was used.

3.5. Immunohistochemistry (II)

To determine the AMACR protein expression, macrosections of 5 µm in thickness were cut from the FFPE (formalin-fixed, paraffin-embedded) blocks of each prostate cross-section (study II), next to the HE-stained sections. Sections were stained with a rabbit monoclonal AMACR (P504S) antibody (1:200, clone 13H4, Zeta Corporation). The detection of primary antibody was performed with EnVision+ Dual Link System-HRP (Dako) and visualized with DAB+ chromogen solution (Dako). The slides were observed

by an experienced uropathologist using a Leica DM3000 light microscope equipped with Leica DFC 420 digital camera and Leica Application Suite version 2.5.0 R1 (Leica Microsystems, Wetzlar).

Table 6. The oligonucleotides used in RT-PCR assays in this doctoral study.

Oligonucleotide	5' ⟶ 3' Sequence	Database sequence number	Position in sequence
ACSM1			
Forward primer	CCAGGAAGTAGGAAATTACG	NM_052956.2	813-832
Reverse primer	GGGTATTTCAACAATGTCTG	NM_052956.2	978-997
Reporter probe	TGTCAAACTGTGGCAGATGGTGGAT AAAGA	NM_052956.2	934-963
Quencher probe	TCTGCCACAGTTTGACA	NM_052956.2	947-963
AMACR			
Forward primer	TTGTCAGGTGTTCTCTCAAA	NM_014324	481–500
Reverse primer	CTTCCACCATATTTGCATC	NM_014324	637–655
Reporter probe	CTGAATCTCCTGGCTGACTTTGCTG G	NM_014324	535–560
Quencher probe	TCAGCCAGGAGATTCAG	NM_014324	535–551
AR			
Forward primer	GCTGAAGGGAAACAGAAGTAC	NM_001011645	328–348
Reverse primer	CTCTCCTTCCTCCTGTAGTTTC	NM_001011645	480–501
Reporter probe	TTGTCGTCTTCGGAAATGTTATGAA GCAGG	NM_001011645	408–437
Quencher probe	AACATTTCCGAAGACGACAA	NM_001011645	408–427
CACNA1D			
Forward primer	AGGAGTGCCCAGTTTACAAG	NM_000720.3	874-893
Reverse primer	TCCTCTTCAGCTACGATATCTG	NM_000720.3	1035-1056
Reporter probe	TTTGTGCATTTTTCCAATAAAAAGTT CCAATCC	NM_000720.3	983-1015
Quencher probe	TTGGAAAAATGCACAAA	NM_000720.3	999-1015
DLX1			
Forward primer	CAATGGCAAGGGAAAAAAG	NM_178120.4	561-579
Reverse primer	GAACCAGATCTTGACCTGAGTC	NM_178120.4	705-726
Reporter probe	TCCAGTTTGCAGTTGCAGGCTTTGA AC	NM_178120.4	604-630
Quencher probe	TGCAACTGCAAACTGGA	NM_178120.4	604-620
KLK3			
Forward primer	AGCATTGAACCAGAGGAGTTCT	X05332	518 – 539
Reverse primer	CCCGAGCAGGTGCTTTTG	X05332	658 – 675
Reporter probe	CCTTCTGAGGGTGAACTTGCGC	X05332	596 – 617
Quencher probe	AAT CAC CCT CAG AAG G	X05332	604 – 617
LMNB1			
Forward primer	ATGTATGAAGAGGAGATTAACG	NM_005573.3	1517-1538
Reverse primer	CATTCTCAAGTTTGGCATG	NM_005573.3	1691-1709
Reporter probe	TCCAGCTCCTCCTTATACAGCCTCA CTT	NM_005573.3	1653-1680
Quencher probe	TGTATAAGGAGGAGCTGGA	NM_005573.3	1662-1680

Oligonucleotide	**5' ⟶ 3' Sequence**	**Database sequence number**	**Position in sequence**
mmPSA			
Forward primer	TGAACCAGAGGAGTTCTTGCA	X05332	523 – 543
Reverse primer	CCCAGAATCACCCGAGCGA	X05332	667 – 685
Reporter probe	CCTTCTGAGGGTGATTGCGCAC	X05332	594 – 601
Quencher probe	AATCACCCTCAGAAGG	X05332	604 – 617
PCA3			
Forward primer	GGTGGGAAGGACCTGATGATAC	Af_103907	95 - 116
Reverse primer	GGGCGAGGCTCATCGAT	Af_103907	505-521
Reporter probe	AGAAATGCCCGGCCGCCATC	Af_103907	478-497
Quencher probe	CCGGGCATTTCT	Af_103907	478-489
PLA2G7			
Forward primer	ATTACAATCAGGGGTTCAGTC	NM_005084.3	1299-1319
Reverse primer	AATCTTTATGAAGTCCTAAATGC	NM_005084.3	1451-1473
Reporter probe	TGCAACTGGCAAAATAATTGGACAC ATGC	NM_005084.3	1346-1374
Quencher probe	TTATTTTGCCAGTTGCA	NM_005084.3	1346-1362
RHOU			
Forward primer	CAAGACGAGCCTGGTGG	NM_021205.5	849-865
Reverse primer	GCTTGTCAAATTCATCCTGTC	NM_021205.5	983-1003
Reporter probe	CCTACTGCCTTCGACAACTTCTCCGC	NM_021205.5	904-929
Quencher probe	TTGTCGAAGGCAGTAGG	NM_021205.5	904-920
SPON2			
Forward primer	GTGACCGAGATAACGTCCTC	NM_012445.3	957-976
Reverse primer	GCGTTTCTGGAACTGAGG	NM_012445.3	1135-1152
Reporter probe	CTGTCTACAATCTCATTGTCCCTGCT GGG	NM_012445.3	1104-1132
Quencher probe	GGACAATGAGATTGTAGACAG	NM_012445.3	1112-1132
TDRD1			
Forward primer	AAAGAAGTGAATATTAAGCCTG	NM_198795.1	523-544
Reverse primer	TAGTAGGTCTGCTTGCACTG	NM_198795.1	700-719
Reporter probe	ACGTCCTGCAAAATCAAAAAAACTA AACAA	NM_198795.1	555-584
Quencher probe	TTTGATTTTGCAGGACGT	NM_198795.1	555-572
TMPRSS2-ERG III			
Forward primer	TAGGCGCGAGCTAAGCAGGAG	NM_005656.3	4-24
Reverse primer	GTAGGCACACTCAAACAACGA	NM_004449.4	338-362
Reporter probe	AGCGCGGCAGGAAGCCTTATCAGTT	NM_005656.3 & NM_004449.4	57-64 & 310-326
Quencher probe	TTCCTGCCGCGCT	NM_005656.3 & NM_004449.4	57-64 & 310-314
TMPRSS2-ERG VI			
Forward primer	CGGCAGGTCATATTGAACATTCC	NM_005656.3	73-95
Reverse primer	GCACACTCAAACAACGACTGG	NM_004449.4	338-358
Reporter probe	CTTTGAACTCAGAAGCCTTATCAGT TGTGA	NM_005656.3 & NM_004449.4	139-149 & 312-330
Quencher probe	GGCTTCTGAGTTCAAAG	NM_005656.3 & NM_004449.4	139-149 & 312-317

3.6. Gene knock-down using RNA interference

PC3 and VCaP prostate cancer cells were used for small interfering RNA (siRNA) studies and other functional studies for 8 of the target genes (*ACSM1*, *TDRD1*, *PLA2G7*, *SPON2*, *DLX1, CACNA1D*, *RHOU*, and *LMNB1*). Cells were grown in RPMI-1640 at 37˚C in standard cell culture conditions (95% humidity and 5% CO_2). For siRNA studies 31 different siRNAs (three different siRNAs for *ACSM1* and four different siRNAs for each of the other seven genes) were ordered from Qiagen (Germany). To achieve the most efficient knock-down for each gene, siRNAs were tested individually and in a pooled mixture. First, siRNAs were plated onto plates, followed by the addition of Hiperfect transfection reagent (Qiagen, Germany) in Opti-MEM medium (Invitrogen, USA). After 15 minutes of incubation at room temperature an appropriate number of the cells were added into each well. ALLStars Hs cell death control siRNA (Qiagen, Germany) was used as positive control for the efficacy of siRNA transfection and ALLStars negative control (Scrambled siRNA, Qiagen, Germany) was used as negative control.

3.7. Cell migration and invasion assay (wound healing)

PC3 transfected cells were plated on 96-well plates and 72 hours after transfection, when cells reached the confluency, a fixed-width wound was scratched with the Woundmaker device (Essen Bioscience, USA).The closure of the wound was quantified and monitored for 72 hours with IncuCyte live-cell imager (Essen bioscience, USA).

3.8. 3D cell culture and image acquisition for morphological analyses

PC3 cells transfected with siRNA were detached from monolayer cultures 72 hours after the transfection. Transfected cells were sandwiched between two layers of Matrigel on uncoated Angiogenesis slides (Ibidi GmbH, Germany). Calcein AM live cell dye was used to stain the resulting tumor organoid cells 10 days after transferring them to the 3D culture medium.

3.9. Data analyses

To determine the threshold cycle (C_t) for each reaction of real-time PCR, a graph was plotted by using signal-to-background ratio against the cycle number. The C_t values were determined visually as the cycle number where the fluorescence level crossed the threshold line and differed significantly from background level. For each concertation of

external DNA standard the difference between the C_t value of the target gene and the C_t value of the reaction containing the maximum yield of internal standard RNA (ΔCt) was calculated. Standard plots were constructed for each target mRNA by plotting ΔCt values against the ten-based logarithm of the external standard concentration. For each unknown sample reaction, ΔCt value was calculated and applied to determine the copy numbers using the standard plot. The obtained copy numbers were normalized to sample size using total RNA amount. After normalization the target mRNA expression level was reported as mRNA copies per µg of total RNA.

Samples were analyzed in triplicates and considered as positive only when all three replicates were positive.

3.10. Statistical analyses

Statistical analyses were performed with SPSS 20.0 (IBM). Nonparametric tests were used to examine the association between clinicopathological parameters and target mRNA expression levels.

4. RESULTS AND DISCUSSION

This doctoral work has mainly focused on evaluating the potency of the mRNA expression of 13 candidate genes as biomarker for PCa diagnosis and prognosis. To be able to choose the most sensitive and specific method for mRNA expression studies by RT-PCR, the applicability of two different labeling chemistries developed on the basis of time-resolved fluorometry (TRF) were assessed.

4.1. Lanthanide chelates and TRF in RT-PCR (I)

The usefulness of gene expression studies by means of microarray and quantitative RT-PCR for cancer classification, and their application in the identification of biomarkers to improve the diagnosis and prognosis of cancer has been reported in numerous studies (Clark-Langone *et al.*, 2007; Scott *et al.*, 2011). Quantitative RT-PCR is known as the gold standard method for quantifying gene expression, and it offers several advantages, such as, high sensitivity, specificity, and reproducibility, as well as accurate quantitation over a wide dynamic range.

Fluorescent-labeled probes designed on the basis of fluorescence resonance energy transfer (FRET) are the most common method used in the detection of PCR products. High background, which is the result of using conventional short-lifetime fluorophores for the labeling of the probes, and autofluorescence from biological sample matrices and the plastic materials of reaction vessels, affects the sensitivity and specificity of RT-PCR assays. Using long-lived luminescent lanthanide chelate probes with time-resolved fluorometry (TRF) has been suggested to overcome this limitation.

In this doctoral work, the applicability of a new reporting chemistry, lanthanide chelate complementation (LCC), in real-time qRT–PCR was investigated and its performance was compared with a previously described hydrolysis enhanced luminescent chelate (HELC) chemistry as a reference method.

The methods were evaluated by measuring the expression level of *KLK3* gene (as model analyte) in prostate tissue and peripheral blood samples taken from cancerous patients and healthy controls. A specific assay based on the LCC method was developed and optimized for measuring *KLK3*, and assay performance was compared with a previously developed *KLK3* assay based on the HELC method. After the optimization of the assays, the same limit of detection was achieved for *KLK3* in standard series (1 copy/µl template) for both assays (Figure 3). The expression level of *KLK3* was measured in three different cDNA dilutions (1:100, 1:1000, and 1:10,000) of 20 tissue samples

collected from 10 PCa patients to investigate the effect of the amount of template cDNA on assay performance.

KLK3 measurement in blood and tissue samples resulted in identical classification in the positive and negative results with both methods. The expression of *KLK3* was detected in all tissue samples with both methods. According to Deming regression analysis results, there was significant correlation for *KLK3* expression between 1:100 and 1:1000 dilutions. However, there was a significant difference in the *KLK3* expression levels for the 1:10,000 dilution between the two detection methods.

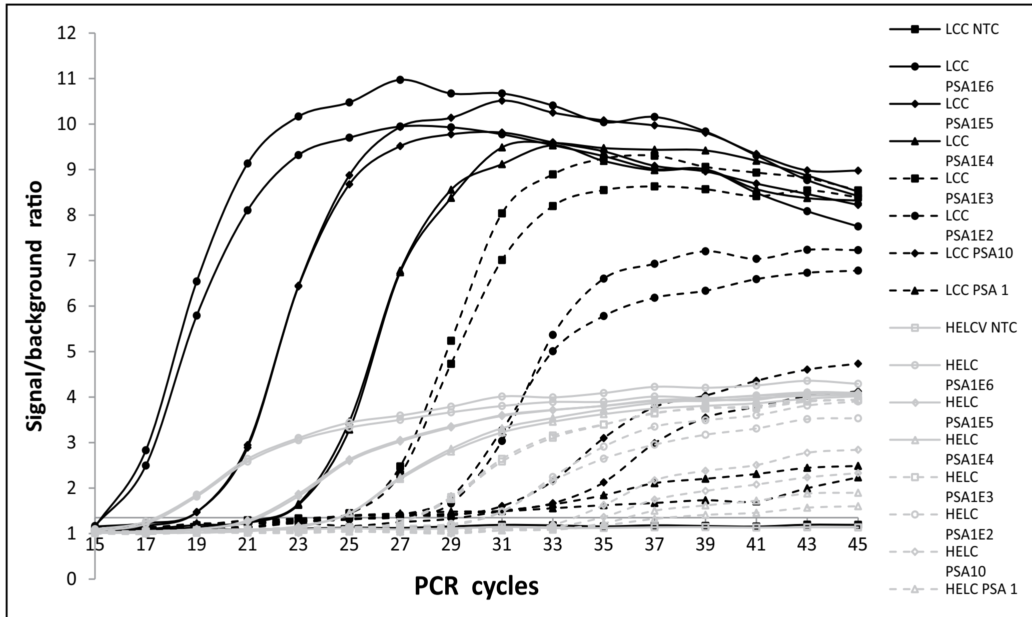


Figure 3. Amplification curve of KLK3 (PSA) standard dilution series (1–106 copies of purified PCR products) and no template control (NTC) with both methods

For mimicking the patient blood samples and CTCs, female blood samples were spiked with different numbers of LNCaP cells. *KLK3* level was measured with both methods. The same limit of detection for the spiked blood samples with LNCaP cells (5 LNCaP cells in 2.5 ml of blood) was obtained with both methods. The expression of *KLK3* was detected only in one similar patient's blood sample with both methods. This could be explained by spiked blood sample results, and the possibility of the number of CTCs in patient's blood samples having been below 5 cells in 2.5 ml of blood.

4.2. Expression of *AMACR* and *AR* in cancerous and noncancerous prostate (II)

In this study, the expression of *AMACR, AR*, and *KLK3* was measured in 138 prostate tissues obtained from 79 men with clinical PCa who had undergone radical prostatectomy, and in 19 Cystoprostatectomy (CP) samples obtained from 19 men with bladder cancer but without clinical PCa. To determine the detailed areas of altered AMACR expression, the AMACR mRNA and protein levels were examined by qRT-PCR and immunohistochemistry, respectively, in whole prostate cross-sections obtained from three RP specimens.

4.2.1. Overexpression of *AMACR*

An overexpression of *AMACR* in PCa and a low to undetectable expression of *AMACR* in normal tissues was reported in 2000 (Xu *et al.*, 2000). AMACR staining using a rabbit monoclonal antibody was reported as a new molecular marker for prostate carcinoma in 2001 (Jiang *et al.*, 2001). In our study, the expression of *AMACR* was detected in all of the 138 RP samples and there was no statistically significant difference between histologically benign and cancerous samples. The expression of *AMACR* was detected in 14/19 CP samples, and a 195-fold increase in the expression of *AMACR* RNA was observed when cancerous radical prostatectomy tissues were compared to the 19 CP samples ($p<0.0001$). The expression of *AMACR* was statistically significantly higher in samples from men with PCa of pathological stage pT3 and pT4 than in samples from men with PCa classified as pT2 ($p=0.006$), but there was no association with the Gleason grades. A comparison of the expression levels of *AMACR* RNA in histologically benign prostate tissues with 19 CP samples also resulted in a 126-fold overexpression ($p<0.0001$) suggesting an alteration of molecular content in areas larger than tumor foci. Increased biomarker expression in histologically benign tissues in itself can be caused by the cancer field effect (Ogden, Cowpe and Green, 1990). The field effect or field cancerization was first suggested by Slaughter *et al.* (Slaughter, Southwick and Smejkal, 1953); nowadays it has an extended definition and is defined as any molecular abnormalities in tissues that appear histologically benign (Hockel and Dornhofer, 2005). To confirm this *AMACR*-related result, three prostate cross-sections obtained from three PCa patients were evaluated in a systematic manner to determine if samples from areas outside the tumor lesions could be equally informative of the presence of cancer. The expression of *AMACR* was detected in all tissue pieces from three prostate sections. There was a 1.9-fold overexpression in median *AMACR* mRNA in cancerous samples compared to histologically benign samples ($p<0.001$). This is in contrast with the result of the RP samples cohort, but it could be explained by considering the fact that all histologically benign (n=112) and cancerous samples (n=35) in the cross-section study

have been collected from a limited number of patients (only three). AMACR staining and immunohistochemical analysis resulted in positive AMACR staining in 100% of areas that contained carcinoma on both the superior and inferior sides of cross-sections of prostate B and C, while on the superior side of prostate A, AMACR staining was observed in 96% of the carcinoma areas. Furthermore, AMACR staining was detected in some of the benign and PIN areas. Positive AMACR staining has been previously reported in 0-21% of histologically normal prostate epithelium (Luo *et al.*, 2002b; Beach *et al.*, 2002; Rubin *et al.*, 2002). This could be explained by the use of different antibodies in different studies, and their sensitivity limitations.

4.2.2. Overexpression of *AR* and *KLK3*

Androgens drive the growth and development of the prostate gland through intracellular androgen receptors (AR). Gene amplification and overexpression of *AR,* that facilitates the growth and maintenance of PCa cells in a very low amount of androgens in castration-resistant PCa, has been reported in a number of previous studies (Koivisto *et al.*, 1997; Visakorpi *et al.*, 1995; Bubendorf, 1999). Here, we studied and compared the expression alteration of *AR* RNA in cancerous prostates (primary PCa) and in noncancerous prostates.

The expression of *AR* and *KLK3* were detected in all of the CP and RP samples. In *KLK3* expression, the gene coding for the PSA did not show any statistically significant differences between different groups of samples. The median RNA expression level of *AR* was 6.4 times higher in the RP-Be samples ($p<0.0001$) and 6.6 times higher in the RP-PCa samples than in CP samples. There was no statistically significant difference in *AR* expression between cancerous radical prostatectomy samples and histologically benign samples. There was no association for the expression of *AR* and *KLK3* with Gleason grade in RP samples, but there was a statistically significant difference for the expression of *AR* and *KLK3* in patients with pathological stage pT3 and pT4 PCa compared with pT2 PCa patients ($p=0.005$ and $p=0.004$, respectively).

The Pearson's correlation coefficient revealed a strong correlation ($r=0.86$) between *AR* and *AMACR* expression levels when samples from all groups were combined. ROC curve analyses for *KLK3*, *AR*, and *AMACR* mRNA expression resulted in AUC values 0.58, 0.717, and 0.923 for *KLK3*, *AR*, and *AMACR* mRNA levels, respectively, when cancerous radical prostatectomy samples were compared with CP samples. The comparison of histologically benign samples with CP samples also resulted in comparable AUC values 0.64, 0.77, and 0.89 for *KLK3*, *AR*, and *AMACR* mRNA levels, respectively (Figure 4).

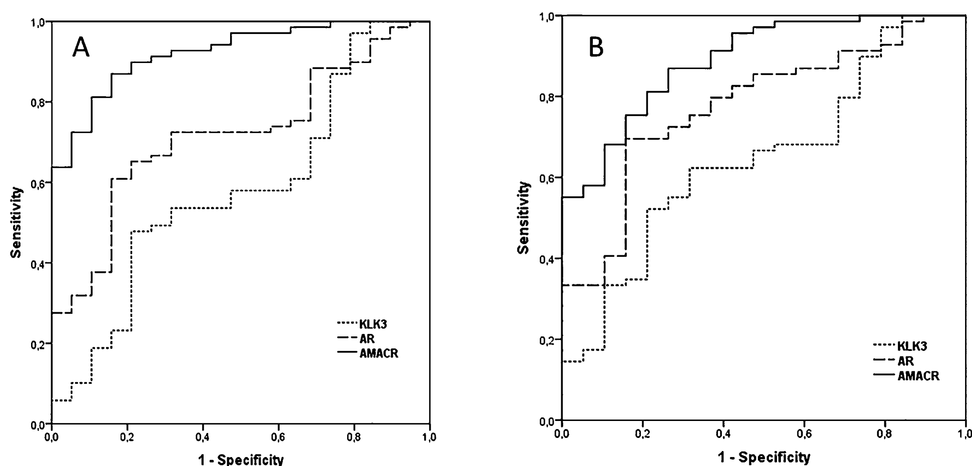


Figure 4. ROC curve analyses for *KLK3*, *AR*, and *AMACR* mRNA expression. A. ROC curve analysis for RP-PCa and CP tissues; AUC values are 0.58, 0.717, and 0.923 for *KLK3*, *AR*, and *AMACR* mRNA levels, respectively. B. ROC curve analysis for RP-Be and CP tissues; AUC values are 0.64, 0.77, and 0.89 for *KLK3*, *AR*, and *AMACR* mRNA levels, respectively. (Figure from original publication II)

The overexpression of *AR* mRNA in both histologically benign and cancerous prostate tissue samples from cancerous prostates compared to samples from prostates without any evidence of PCa confirms the previous study results that PCa is dependent on the androgen/AR signaling pathway in all stages of the disease and not only in the metastatic stage (Chen *et al.*, 2004; Isaacs, 1994; Linja *et al.*, 2004). Furthermore, on the basis of *AMACR* mRNA overexpression in histologically benign samples, and comparable AUC values for *AMACR* mRNA expression between histologically benign and cancerous samples, the usefulness of an *AMACR* mRNA measurement with a qRT-PCR assay for patients with false negative biopsies (i.e., lesion missed in the biopsies) in assessing their cancer risk could be considered.

4.3. Identification and validation of novel biomarkers for PCa (III)

Applying both different filtering and approaches for mining the databases in order to identify specific biomarkers for the stratification and discrimination of primary PCa either from a normal prostate or from metastasized PCa resulted in eight candidate genes (*ACSM1, AMACR, CACNA1D, DLX1, PLA2G7, RHOU, SPON2*, and *TDRD1*) for further clinical and functional validations.

4.3.1. Evaluation of the mRNA expression of candidate genes in tissue samples

The expression of eight selected candidate genes in addition to *KLK3* was measured in a tissue cohort including 178 prostate tissues obtained from 90 men with clinical PCa

and 19 previously mentioned CP samples. Expression levels and the frequency of detection of target mRNAs in different groups of samples are shown in Figure 5 and Table 7.

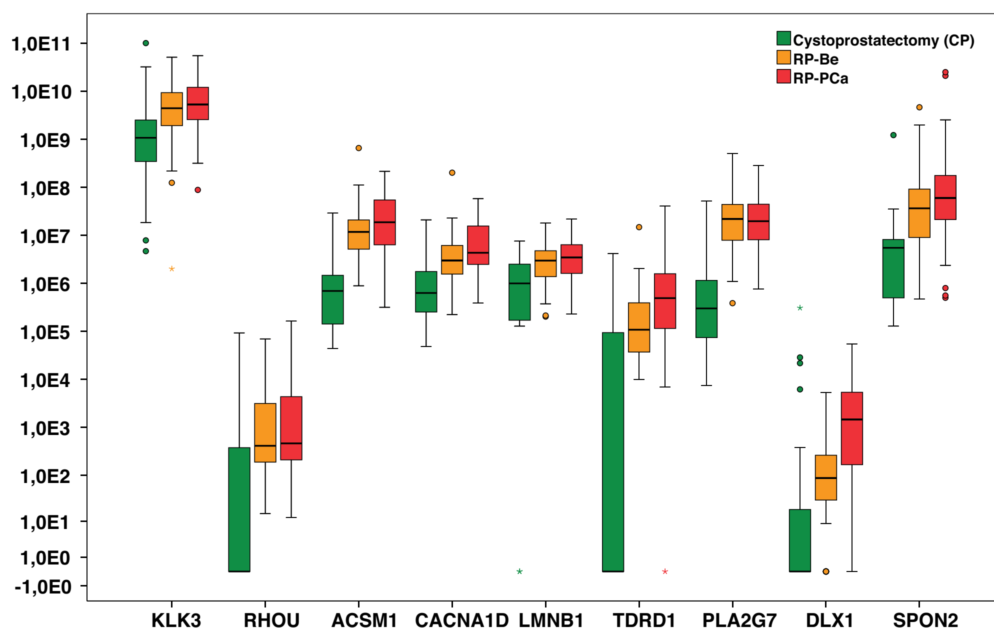


Figure 5. Expression levels of eight candidate biomarker genes (mRNA copies/µg of total RNA) and *KLK3* in cystoprostatectomy samples (CP), histologically benign radical prostatectomy samples (RP-Be), and cancerous radical prostatectomy samples (RP-PCa). Boxes show the interquartile range, with the line in the middle denoting the median value and circles representing the outliers. (Figure from original publication III)

Table 7. Frequency of detection of target mRNAs in different tissue samples.

Number and percentage of samples where target mRNA was detected				
Target mRNA	CP-B samples(n=7)	CP-IPCa samples (n=12)	RP-B samples (n=104)	RP-PCa samples (n=74)
KLK3	7 (100%)	12 (100%)	104 (100%)	74 (100%)
LMNB1	5 (71%)	12 (100%)	104 (100%)	74 (100%)
ACSM1	7 (100%)	12 (100%)	104 (100%)	74 (100%)
CACNA1D	7 (100%)	12 (100%)	104 (100%)	74 (100%)
RHOU	2 (28%)	7 (58%)	104 (100%)	74 (100%)
DLX1	0 (0%)	5 (41%)	88 (85%)	73 (99%)
TDRD1	2 (28%)	6 (50%)	101 (97%)	72 (97%)
PLA2G7	7 (100%)	12 (100%)	104 (100%)	74 (100%)
SPON2	7 (100%)	12 (100%)	104 (100%)	74 (100%)

(CP-B samples) indicates benign prostate tissue from patients without PCa, (CP-IPCa samples) indicates benign prostate tissue from patients with incidental PCa, (RP-B samples) indicates benign prostate tissue from patients with PCa, and (RP-PCa) indicates cancerous tissue from patients with PCa. (Table from original publication III)

The p values of the comparison of differential expression levels between different groups of samples and the association of mRNA expression of target genes in prostate tissue with major clinical and pathological parameters of PCa has been listed in Table 8.

Table 8. Association of mRNA expression of target genes with clinicopatholocical parameters. (Table from original publication III)

	Target mRNA								
	KLK3	*RHOU*	*ACSM1*	*CACNA1D*	*LMNB1*	*TDRD1*	*PLA2G7*	*DLX1*	*SPON2*
CP (n=19) vs. RP-B (n=104)	0.008	<0.001	<0.001	<0.001	0.001	0.001	<0.001	0.008	<0.001
CP (n=19) vs. RP-PCa (n=74)	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
RP-PCa (n=74) vs. RP-B (n=104)	0.7	0.30	0.001	0.001	0.14	<0.001	0.804	<0.001	0.00
Prostate Gleason ≥ 7 (n=43) vs. Prostate Gleason ≤,6 (n=43)*	0.866	0.249	0.849	0.799	0.331	0.115	0.048	0.074	0.707
pT 2 (n=51) vs. pT 3,4 (n=35)*	0.161	0.535	0.833	0.004	0.072	0.377	0.715	0.001	0.775
PSA relapse (n=15) vs. No PSA relapse (n=65) *	0.225	0.980	0.206	0.090	0.151	0.209	0.427	0.156	0.023

* A single value of mRNA expression of each gene, was used (consistently from the right lobe the prostate) for each patient with two cancerous samples

The comparison of all 19 cystoprostatectomy samples (CP) with either histologically benign (RP-B) or cancerous radical prostatectomy (RP-PCa) samples resulted in statistically significant p values for all the target genes, while only the expression of *ACSM1, CACNA1D, DLX1, SPON2*, and *TDRD1* was capable to discriminate RP-PCa samples from RP-B samples. To investigate the association of mRNA expression of target genes in prostate tissue with major clinical and pathological parameters of PCa, for each patient with two cancerous samples a single value of mRNA expression of each gene, was used (consistently from the right lobe of the prostate). Except for *PLA2G7* (p=0.048), none of the other target genes were able to discriminate aggressive tumors (Gleason score ≥7) from less aggressive PCa (Gleason score <7) on their own. The expression of *CACNA1D* and *DLX1* were significantly different between T2 and T3 categories of TNM staging (p=0.004 and 0.001, respectively). Only the expression of *SPON2* (p=0.023) was able to distinguish between patients with or without PSA relapse.

The ROC analysis was used to evaluate the sensitivity and specificity of candidate biomarkers (Figure 6). A comparison of all 19 CP samples (considered as negative) with cancerous radical prostatectomy samples (considered as positive; only one sample from patients with two cancerous samples was utilized) resulted in area under the curve (AUC) values varied between 0.74 and 0.93 (Table 9). Furthermore, a comparison of CP samples (considered as negative) with histologically benign radical prostatectomy samples (considered as positive) using a single value of mRNA expression of each gene for each patient resulted in comparable AUC values varied between 0.69 and 0.92 (Table 9). The similar predictive power of these genes using either histologically benign or

cancerous radical prostatectomy samples could be explained by the cancer field effect and molecular alterations in tissue samples that appear histologically benign.

Table 9. The table presents the AUC values for each gene in the ROC analyses.

(C) Area Under the Curve table		
Target mRNA	RP-PCa (only one sample per patient, n=59) vs. CP (n=19)	RP-Be (only one sample per patient, n=74) vs. CP (n=19)
KLK3	0.740	0.709
RHOU	0.773	0.761
ACSM1	0.921	0.910
CACNA1D	0.839	0.784
LMNB1	0.789	0.751
TDRD1	0.830	0.747
PLA2G7	0.935	0.927
DLX1	0.783	0.698
SPON2	0.863	0.804

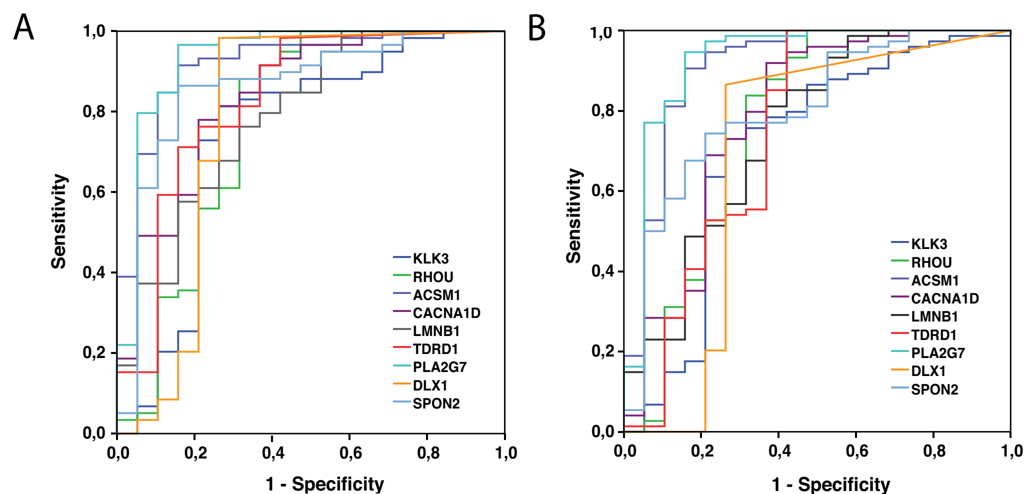


Figure 6. ROC analyses for *KLK3* mRNA levels and expression levels of 8 target mRNAs with cases classified as having cancer or not. To simplify the analyses for the patients with two cancerous/benign samples, a single value of mRNA expression of each gene for each patient, consistently from the right lobe of the prostate, was chosen. A. RP-PCa samples were considered as positive samples and compared with all CP samples (defined as negative); high sensitivity and specificity was observed for all eight biomarkers. B. RP-Be samples were considered as positive samples and compared with all CP samples (defined as negative). (Figure from original publication III)

A statistically significant difference was observed for candidate gene expression between normal prostate samples and malignant PCa samples. On the basis of clinical validation results and the association of the expression of target genes with clinicopathological parameters, *PLA2G7, CACNA1D, DLX1*, and *SPON2* would be the

most informative for prognostic applications. An application of *SPON2* as a novel biomarker for the detection of PCa and CRPC has recently been reported (Lucarelli *et al.*, 2013; Barbieri, 2013; Qian *et al.*, 2012). Furthermore, the overexpression of *PLA2G7* and *TDRD1* in PCa and the association of *PLA2G7* with aggressive forms of PCa have been reported earlier (Vainio *et al.*, 2011a; Vainio *et al.*, 2011b; Shaikhibrahim *et al.*, 2013; Massoner *et al.*, 2013; Bertilsson *et al.*, 2012).

4.3.2. Knockdown with siRNA transfection and its effects on invasion and motility of PC-3 cells

Compared to LNCaP and VCaP cells, a higher knockdown efficacy was observed in PC-3 cells. On the basis of *in silico* analysis results, all of the selected candidate genes, with the exception of *SPON2*, were expressed in PC-3 cells. Hiperfect was used as transfection reagent, and qRT-PCR measurement revealed 70-95% knockdown levels for different genes either individually or combined in a siRNA pool. For all the eight genes, with the exception of *ACSM1*, a single siRNA, superior to pooled siRNA, was shown to be the most effective for transfection. The most effective siRNA (single or pooled), was selected for all further experiments.

The comparison of wound healing experiment results between PC-3 cells transfected by different genes' siRNA and either scrambled siRNA-transfected or untreated (mock transfected) PC-3 cells revealed a strong and reproducible effect on cell motility and migration only for *RHOU* gene. A stimulation of the T-ALL cell migration by an upregulation of *RHOU* that could contribute to enhanced motility and dissemination of leukaemia cells has been previously reported (Bhavsar *et al.*, 2013). Similar effects could promote the aggressive and invasive properties of advanced PCa.

4.3.3. Effect of gene knockdown on PC-3 cell morphology in 3D model

Ten days after transferring the cells to a 3D culture, the resulting tumor organoid cells were stained with Calcein AM live cell dye (ThermoFisher, USA) and ethidium homodimer 1 (EthD-1) that stains dead and apoptotic cells. Confocal images were then taken using a spinning disc. After noise filtering and image segmentation, a total of 26 different morphological parameters were evaluated using Automated Morphometric Image Data Analysis (AMIDA) (Harma *et al.*, 2014). The parameters are particularly informative for the growth, differentiation, invasive properties, and multicellular organization of tumor structures. The effects of knockdown by siRNA on five of the more important parameters (area, density, MaxApp, roughness, and roundness) were investigated.

ACSM1 (acyl-CoA synthetase medium-chain family member 1) has not been previously associated with PCa progression. Silencing *ASCM1* resulted in weak morphologic effects and a mild reduction of invasive features (increased area and roundness and

decreased roughness compared to scrambled samples). *ACSM1* has been reported as a potential marker for the invasive apocrine subtype of breast cancer which is associated with AR+ status and poor differentiation (Bockmayr *et al.*, 2013; Celis *et al.*, 2008; Celis *et al.*, 2009).

CACNA1Ds (calcium channel, voltage-dependent, L-type, alpha 1D subunits) are calcium channels involved in the entry of calcium ions into the cells and control a variety of calcium-dependent processes, such as gene expression. The role of *CACNA1D* in PCa cell growth promotion and proliferation (Chen *et al.*, 2014) and in castration-resistant cancer progression (Loughlin, 2014) has been previously reported.

In our study, the silencing of *CACNA1D* resulted in morphologic effects including the blocking of invasive structures and growth inhibition (increased area and roundness, and decreased roughness and MaxApp, compared to cells treated with scrambled siRNA).

DLX1 (distal-less homeobox 1) gene encodes a transcription factor similar to the Drosophila distal-less gene. An overexpression of *DLX1* in PCa has been published (Leyten *et al.*, 2015; Pascal *et al.*, 2009; Altintas *et al.*, 2013) and it was confirmed by our clinical validation results. The silencing of *DLX1* increased the cell density and roundness, but it decreased the area, MaxApp, and roughness parameters in comparison to cells treated with scrambled siRNA.

LMNB1 (lamin B1) is a member of the lamin family that are highly conserved in evolution and involved in nuclear stability, chromatin structure, and gene expression. A reduced protein expression of LMNB1 in colon cancers (Moss *et al.*, 1999) and a declined mRNA expression of *LMNB1* in association with poor clinical outcome in breast cancer have been reported (Wazir *et al.*, 2013). In our work, we found that the expression of *LMNB1* is increased in PCa samples compared to noncancerous samples. Furthermore, an overexpression of *LMNB1* in human pancreatic cancer and its association with an increased incidence of distant metastasis and poor prognosis has been previously reported (Li *et al.*, 2013). An analysis of AMIDA parameters revealed that *LMNB1* silencing results in increased area, roundness, and MaxApp and decreased roughness compared to cells treated with scrambled siRNA.

PLA2G7 (phospholipase A2, group VII) also known as PAFAH (plasma platelet-activating factor acetylhydrolase) is involved in the hydrolysis of phospholipids into fatty acids and other lipophilic molecules. As the association of *PLA2G7* with aggressive PCa and its usefulness as a biomarker and drug target has been reported earlier (Vainio *et al.*, 2011b; Bertilsson *et al.*, 2012), it was selected as a positive control gene for our study. Silencing *PLA2G7* in PC-3 cells resulted in increased density and roundness and decreased area, MaxApp and roughness compared to PC-3 cells treated with scrambled siRNA.

RHOU (ras homolog family member U) is a member of the Rho family of GTPases that acts upstream of PAK1 regulating the actin cytoskeleton and adhesion turnover, and increasing cell migration (Ory, Brazier and Blangy, 2007). The association of *RHOU* with PCa progression has not been reported earlier. In this study, the silencing of *RHOU* in PC-3 cells resulted in increased area, roundness and density, and decreased MaxApp and roughness when compared with PC-3 cells treated with scrambled siRNA.

SPON2 (spondin 2) is an extracellular matrix protein-coding gene. *SPON2* is not expressed in PC-3 cells, and was therefore used as a negative control; there was no difference in selected parameters between *SPON2* knockdown and scrambled siRNA-treated PC-3 cells.

The usefulness of measuring *TDRD1* (tudor domain containing 1) expression in urine samples in the early diagnosis of patients with clinically significant PCa has been reported (Leyten *et al.*, 2015). Furthermore, the role of *TDRD1* promoter methylation and its usefulness as an independent predictor of biochemical recurrence in high-risk PCa patients has been reported (Litovkin *et al.*, 2014). In our study, the silencing of *TDRD1* gene in PC-3 cells resulted in increased area and MaxApp, and decreased density and roundness in comparison with PC-3 scrambled siRNA treated PC-3 cells.

The highest cytotoxic phenotypic effects were observed upon silencing *DLX1* and *PLA2G7*, which resulted in a characteristic and significantly decreased area, roughness, and MaxApp in addition to an increased roundness and density compared to scrambled control-transfected cells. Taken together, these results indicate a generation of small, poorly proliferative, and non-invasive organoids that are essentially growth-arrested. Furthermore, the silencing of *RHOU, ACSM1*, and *CACNA1D* resulted in a noticeable, but not entirely specific inhibition of pro-invasive features (reduced MaxApp and roughness), combined with a strongly increased roundness; however, a less prominent reduction of cell growth (area) was observed.

The results of this study provide evidence for these eight candidate genes as potential biomarkers in the diagnosis and prognosis of PCa. Furthermore, the functional study results revealed the putative significance of these genes in disease-relevant processes.

4.4. Expression of 11 biomarker genes in apparently benign tissue samples (IV)

In this study we investigated the informative value of measuring 11 genes' expression levels by qRT-PCR in biopsy cores retrieved from apparently non-cancerous areas. The genes were selected on the basis of the results in our previous studies that showed statistically significant differences in gene expression in histologically benign tissues

from men with and without PCa. The expression of *ACSM1, AMACR, CACNA1D, DLX1, PCA3, PLA2G7, RHOU, SPINK1, SPON2, TMPRSS2-ERG*, and *TDRD1* in addition to *KLK3* (as positive control) was measured in 99 prostate biopsies taken from apparently benign areas (as targeted by MRI) from men with a clinical suspicion of PCa.

The expression of *ACSM1, AMACR*, *CACNA1D*, *KLK3*, *PLA2G7*, *SPON2*, and *TDRD1* RNAs were detected in all 99 samples, whereas *PCA3* and *SPINK1* were detected in 96% of the samples from men without PCa and 100% and 98% of the samples from men with PCa, respectively; *RHOU*, *DLX,* and *TMPRSS2-ERG* mRNAs were detected less frequently. The expression of *KLK3* was the highest among the 11 candidate genes and the expression levels of *DLX1* and *TMPRSS2-ERG* were the lowest (Figure 7). Only the expression of *TDRD1* showed a statistically significant difference between men with and without a PCa diagnosis (p=0.029). Furthermore, Fisher's exact test revealed a significant difference in the detection of *TMPRSS2-ERG* (any variant) between men with and without PCa (p=0.035).

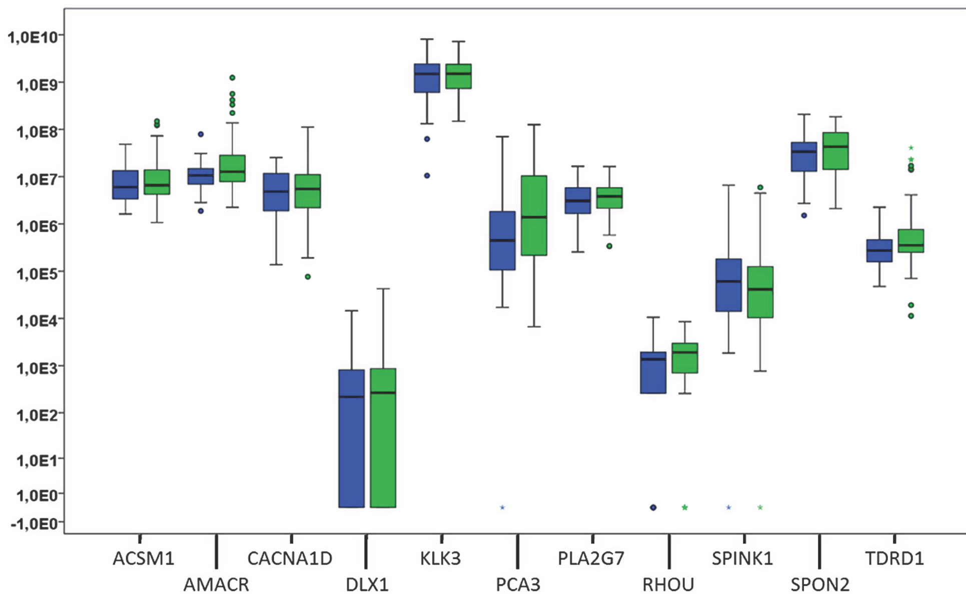


Figure 7. Expression levels of 11 genes in biopsies from men with (green boxes) and without (blue boxes) a diagnosed PCa at biopsy. Boxes show the interquartile range, with the line in the middle denoting the median value. (Figure from original publication IV)

Furthermore, the expression of *TDRD1* was statistically significant when men with aggressive PCa (patients with Gleason sum 7–10 at biopsy) were compared with men with low-risk PCa (Gleason sum < 7 at biopsy). Moreover, the expression of *TMPRSS2-*

ERG mRNAs was detected in 26/53 (49.1%) aggressive cases and in 6/46 (13.0%) less aggressive or healthy cases (Fisher's exact test, $p<0.001$). The percentage of free PSA in serum showed a statistically significant difference in men with PCa vs. men without (Mann-Whitney U test, $p<0.001$). Furthermore a statistically significant difference was observed for the percentage of free PSA in serum, in men with PCa of Gleason sum 7 or higher vs. men with lower risk PCa or no PCa (Mann-Whitney U test, $p=0.002$). There was no significant difference in total PSA between the above-mentioned groups. There was no statistically significant difference for any of the candidate genes' expressions between men with pT2 stage and men with pT3 stage of PCa.

As the reason of choosing these 11 genes for this study was their capability to discriminate between histologically benign tissues from men with and without PCa, the fact that only two of the candidate RNAs showed such capability in this study is in contrast with our hypothesis. This could be explained by differences in samples without PCa that were used in these studies. Here, biopsies from individuals with clinical suspicion of PCa were used, but in previous studies cystoprostatectomy samples obtained from men with no clinical suspicion of PCa were used as non-cancerous cases. Another possibility could be the presence of microfoci of cancer in the biopsy core that remained undetected in MRI and systematic biopsies. Moreover, using different methods and RNA preservative solutions (the commercial RNA stabilizing solution, RNAlater, in this study compared to fresh-freezing and GITC solution used in previous studies) could have caused the differences in the results.

The improvement of the diagnosis and prognosis potency of single biomarkers by combining them together and using a multivariate analysis has been previously reported (Cuzick *et al.*, 2011; Erho *et al.*, 2013; Wu *et al.*, 2013). In accordance to these studies, we found that a combination of 11 target genes with clinical data (serum PSA, F/T PSA percentage, and prostate volume) and a multivariate analysis using logistic regression classifier with L2-norm regularization (Buitinck *et al.*, 2013) and leave-pair-out cross-validation (LPOCV) (Airola *et al.*, 2011) improved the potency of discrimination between aggressive PCas and non-aggressive PCas, and non-cancerous prostates, as well as T3 PCas and T2 PCas or non-cancerous prostates, compared with analyses utilizing each of the 15 features individually (Table 10).

The transcriptional activation of *TDRD1* in *TMPRS2-ERG* positive PCa and the association of upregulation of *TDRD1* with the overexpression of ERG in primary PCa have been previously reported (Kacprzyk *et al.*, 2013; Boormans *et al.*, 2013). In this study, we found that *TDRD1* mRNA levels were 1.8 times higher in *TMPRSS2-ERG* positive samples (Mann-Whitney U test, $p=0.003$).

Table 10. Area under the curve (AUC) values of receiver operating characteristic (ROC) analyses for prostate volume, serum PSA levels, free-to-total PSA ratios in serum, and levels of 12 target mRNAs in tissue individually and in multivariate analyses. The analyses included those 80 men for whom all 15 features were available. Cases were classified according to disease aggressiveness and invasion. Clinically significant aggressive PCa was defined as Gleason sum 7 or higher at biopsy.

	Area under the curve	
Measure	**PCa of Gleason sum 7 or higher vs No clinically significant disease**	**T3 vs T2 and healthy**
Prostate volume	0.646	0.746
Total PSA in serum	0.566	0.743
F/T[1] PSA % in serum	0.703	0.609
ACSM1 mRNA	0.501	0.501
AMACR mRNA	0.520	0.532
CACNA1D mRNA	0.575	0.562
DLX1 mRNA	0.518	0.584
KLK3 mRNA	0.534	0.511
PCA3 mRNA	0.560	0.550
PLA2G7 mRNA	0.555	0.586
RHOU mRNA	0.521	0.539
SPINK1 mRNA	0.514	0.586
SPON2 mRNA	0.521	0.519
TDRD1 mRNA	0.649	0.581
Any detectable *TMPRSS2-ERG* mRNA	0.683	0.572
Multivariate LPOCV* using clinical parameters (total PSA, F/T PSA% and prostate volume)	0.684	0.867
Multivariate LPOCV* using 15 features	0.767	0.818

5. CONCLUSIONS

This doctoral work was planned to identify novel diagnosis and prognosis biomarkers for PCa. In this thesis, the potential of bioinformatic tools, microarray datasets, qRT-PCR, and siRNA technique were combined to identify and validate novel biomarkers for PCa.

It can be concluded on the basis of the original publications that:

I. Applying LCC reporter probes as a new detection method provides the same sensitivity and specificity as the previously reported HELC technique. The use of either LCC or HELC method resulted in the same limit of detection for *KLK3* standard series (1 copy/µl template). Quantifiable levels of KLK3 mRNA were seen in all of the prostate tissue samples, whereas this was true for only one of the blood samples from PCa patients. Therefore, it could be concluded that blood samples from patients with a localized PCa are not suitable for CTC and gene expression studies.

II. Compared to CP samples, *AMACR* mRNA expression was similarly upregulated in all of the RP-Be and RP-PCa samples, which indicates a global overexpression of *AMACR* in prostates with carcinoma. The overexpression of AMACR in histologically benign areas of prostates harboring cancer was also detected in the preliminary, but systematic, cross-section study here. Therefore, it could be concluded that an *AMACR* mRNA measurement with a qRT-PCR assay could be useful for patients with false negative biopsies when assessing their cancer risk.

III. The knockdown by siRNA resulted in a cytotoxic effect for *DLX1, PLA2G7*, and *RHOU* silencing; furthermore, a cell invasion alteration was caused by *PLA2G7, RHOU, ACSM1*, and *CACNA1D* silencing in 3D, as well as cell-motility blocking effects by *PLA2G7* and *RHOU* silencing in 2D. A further statistically significant mRNA overexpression was observed for all eight genes in malignant PCa samples compared to normal prostate samples. Additionally, significant differences were observed in low versus high Gleason grades (for *PLA2G7*), PSA relapse versus no relapse (for *SPON2*), and low versus high TNM stages (for *CACNA1D* and *DLX1*). These results indicate the usefulness of bioinformatic data mining in combination with clinical biomarker validation by qRT-PCR and the functional evaluation of candidate genes in the identification of novel disease-relevant biomarkers. The eight novel biomarkers studied here show the potential to become useful biomarkers for PCa diagnosis. Furthermore, the possibility of using some of these genes as therapeutic targets could be considered in future studies.

IV. Measuring the mRNA expression of *TDRD1* and *TMPRSS2-ERG* as single markers in apparently benign prostate tissue biopsies from men with a clinical suspicion of PCa was useful in discriminating between men with and without PCa. However, utilizing a multivariate analysis using 12 genes' RNA expression in combination with serum PSA, F/T PSA, and prostate volume outperformed each individual marker in distinguishing aggressive PCa from indolent disease. This result provides evidence that multiplex approaches and multivariate analyses may improve the diagnostic and prognostic potency of the markers in comparison with the use of a single biomarker.

Our results support the previous findings that a single biomarker does not provide sufficient sensitivity and specificity for PCa diagnosis. Combining several different RT-PCR assays into a single multiplex RT-PCR reaction is challenging, and a careful design and optimization of the assays are necessary. The optimization and validation of the assays could be costly and time consuming. Increasing the number of the targets will increase the cost and time involved. Furthermore, due to the limited number of available filters in RT-PCR instruments only a limited number of the target genes could be included. An alternative for multiplex approach would be the application of the statistical models and multivariate analyses to combine the separate assay results into an informative method. Several studies have investigated the predictive value of different gene expression panels in PCa. A genomic classifier including 22 genes that predicts the early metastasis of PCa following radical prostatectomy is commercially available by Decipher ® (USA) (Erho *et al.*, 2013). There are also several other commercially available gene expression panel tests by Prolaris® (USA) for the prediction of metastatic progression, mortality, biochemical recurrence, survival, and treatment failure (Cuzick *et al.*, 2011; Cooperberg *et al.*, 2013; Cuzick *et al.*, 2012; Bishoff *et al.*, 2014; Freedland *et al.*, 2013).

AKNOWLEDGMENTS

This study was carried out at the Division of Biotechnology at the University of Turku, Finland during 2010-2016. The work was supported by European Union (FP-7, Marie Curie) under the Prostate Research Organizations-Network of Early Stage Training (PRO-NEST, Project reference: 238278) and by the doctoral program in molecular life science of the University of Turku.

During this time I have had the opportunity to perform this research with highly skilled professionals. I wish to express my deepest gratitude to my supervisors, Professor Kim Pettersson, Dr. Riina-Minna Väänänen, and Dr. Matthias Nees for all their support and advice throughout this thesis project. Their immense knowledge, encouragement and patience provided motivation and confidence to carry out this PhD work.

I would like to thank Dr. Gerald Verhaegh and Professor Craig Robson for all their time and reviewing this thesis. Their excellent comments and feedback helped to improve this work. I also gratefully appreciate the work of Anu Toivonen who revised the language of this thesis book.

I want to sincerely thank all the co-authors of the original publications and manuscripts. Dr. Ari Lehmusvuori, Dr. Ulla Karhunen, Professor Tero Soukka, Natalia Tong Ochoa, Dr. Emily A Vertosick, Terhi Tallgrén, Ileana Montoya Perez, Dr. Ivan Jambor, Professor Hannu Aronen, Jesse Mattsson, Yifeng Li, and Malin Åkerfelt – all your contribution and hard work made this study and publications possible. The clinical collaborators Dr. Esa Kähkönen, Dr. Pekka Taimen, Docent Kalle Alanen, Docent Peter J Boström, Dr. Otto Ettala, Dr. Kari Syvänen, and Dr. Markku Kallajoki at Turku University Hospital in Turku and Professor Anders Bjartell at Skåne University Hospital in Malmö, Sweden are gratefully acknowledged for providing specimens for this study as well as their expertise and advices during this study.

I would also like to thank all my co-workers at the Division of Biotechnology in particular Dr. Mari Peltola, Henna Kekki, Ferdhos Khan Liton, Erica Kuusela and Minna Soikkeli for their help, support and encouragement. I am grateful to all members of PRO-NEST project, particularly Dr. Ellen Schenk and Professor Guido Jenster for a memorable network which made it possible to initiate the collaboration with several research centers.

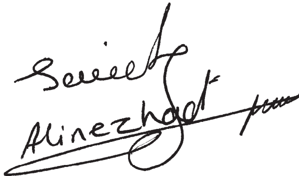
I would like to take this opportunity to thank my best friends in Turku Mohammad Khajeheian, Reza Zahiri, Mehdi Farshchian, Amir Rahmani, Sanaz Rahimi, Amir Shirdel, Parisa Movahedi, Mehrbod Mohammadian, Tolou Shadbahr, Ramin Chavoshi, Parisa Karbalayi, Hamed Mehr, Shohreh Hosseinzadeh, Sina Tadayon, Mohammad

Kafami, Akhgar Kaboli, and Helena Levola for help, support and all the warm, great and fun moments that we had together: your friendship made it possible to get through the difficult times. I would also like to thank Hamid Mirzayi and Hamid Abbasi, my old friends from the time of my bachelor studies and Dr. Kamran Varshokar, for encouragement and long friendship.

My dear Thanea, thanks for all lovely days during the last two years and for being nice and patient. I would also like to thank your family for welcoming me so warmly into your company.

My most sincere and warmest thanks belong to my family. My dear parents Arshad and Habibeh, thank you for your love, endless support and the encouragement given to me toward education and excellence; nothing would have been possible without your unconditional help. My dear sisters Hamideh, Masoomeh, and Azar: thanks for believing in me, for always being with our parents and taking care of my responsibilities - I am forever grateful. I also extend my gratitude to my brothers-in-law, Mohammad Reza and Arash for all their help and support.

Turku, March 2016



Saeid Alinezhad

REFERENCES

Airola, A., T. Pahikkala, W. Waegeman, B. De Baets, *et al.* (2011) An experimental comparison of cross-validation techniques for estimating the area under the ROC curve. *Computational Statistics & Data Analysis,* 55**,** 1828-1844.

Altarac, S. (2013) [Individualized "opportunistic" screening for prostate cancer instead of mass population screening]. *Lijec Vjesn,* 135**,** 51-2.

Altintas, D. M., N. Allioli, M. Decaussin, S. de Bernard*, et al.* (2013) Differentially Expressed Androgen-Regulated Genes in Androgen-Sensitive Tissues Reveal Potential Biomarkers of Early Prostate Cancer. *Plos One,* 8.

Altman, D. G., L. M. McShane, W. Sauerbrei & S. E. Taube (2012) Reporting recommendations for tumor marker prognostic studies (REMARK): explanation and elaboration. *Bmc Medicine,* 10.

Arem, H., S. T. Mayne, J. Sampson, H. Risch*, et al.* (2013) Dietary fat intake and risk of pancreatic cancer in the Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial. *Ann Epidemiol,* 23**,** 571-5.

Barbieri, C. E. (2013) Evolution of Novel Biomarkers for Detection of Prostate Cancer. *Journal of Urology,* 190**,** 1970-1971.

Barqawi, A. B., K. O. Rove, S. Gholizadeh, C. I. O'Donnell*, et al.* (2011) The role of 3-dimensional mapping biopsy in decision making for treatment of apparent early stage prostate cancer. *J Urol,* 186**,** 80-5.

Baum, M. (2013) Screening for prostate cancer: can we learn from the mistakes of the breast screening experience? *Eur Urol,* 64**,** 540-1.

Beach, R., A. M. Gown, M. N. de Peralta-Venturina, A. L. Folpe*, et al.* (2002) P504S immunohistochemical detection in 405 prostatic specimens including 376 18-gauge needle biopsies. *American Journal of Surgical Pathology,* 26**,** 1588-1596.

Belbase, N. P., C. S. Agrawal, P. K. Pokharel, S. Agrawal*, et al.* (2013) Prostate cancer screening in a healthy population cohort in eastern Nepal: an explanatory trial study. *Asian Pac J Cancer Prev,* 14**,** 2835-8.

Bertilsson, H., M. B. Tessem, A. Flatberg, T. Viset*, et al.* (2012) Changes in Gene Transcription Underlying the Aberrant Citrate and Choline Metabolism in Human Prostate Cancer Samples. *Clinical Cancer Research,* 18**,** 3261-3269.

Bhatia-Gaur, R., A. A. Donjacour, P. J. Sciavolino, M. Kim*, et al.* (1999) Roles for Nkx3.1 in prostate development and cancer. *Genes Dev,* 13**,** 966-77.

Bhavsar, P. J., E. Infante, A. Khwaja & A. J. Ridley (2013) Analysis of Rho GTPase expression in T-ALL identifies RhoU as a target for Notch involved in T-ALL cell migration. *Oncogene,* 32**,** 198-208.

Bill-Axelson, A., L. Holmberg, M. Ruutu, H. Garmo*, et al.* (2011) Radical prostatectomy versus watchful waiting in early prostate cancer. *N Engl J Med,* 364**,** 1708-17.

Bishoff, J. T., S. J. Freedland, L. Gerber, P. Tennstedt*, et al.* (2014) Prognostic Utility of the Cell Cycle Progression Score Generated from Biopsy in Men Treated with Prostatectomy. *Journal of Urology,* 192**,** 409-414.

Bockmayr, M., F. Klauschen, B. Gyorffy, C. Denkert*, et al.* (2013) New network topology approaches reveal differential correlation patterns in breast cancer. *BMC Syst Biol,* 7**,** 78.

Boormans, J. L., H. Korsten, A. J. C. Ziel-van der Made, G. J. L. H. van Leenders*, et al.* (2013) Identification of TDRD1 as a direct target gene of ERG in primary prostate cancer. *International Journal of Cancer,* 133**,** 335-345.

Bostwick, D. G. (2000) Prostatic intraepithelial neoplasia. *Curr Urol Rep,* 1**,** 65-70.

Boutros, P. C., M. Fraser, N. J. Harding, R. de Borja*, et al.* (2015) Spatial genomic heterogeneity within localized, multifocal prostate cancer. *Nature Genetics,* 47**,** 736-+.

Brawer, M. K. (2005) Prostatic intraepithelial neoplasia: an overview. *Rev Urol,* 7 Suppl 3**,** S11-8.

Brooks, D. D. (2013a) Prostate cancer screening in black men--new questions, few answers. *Cancer,* 119**,** 2206-8.

Brooks, J. D. (2013b) Managing localized prostate cancer in the era of prostate-specific antigen screening. *Cancer,* 119**,** 3906-9.

Brooks, J. D., G. S. Bova, C. M. Ewing, S. Piantadosi*, et al.* (1996) An uncertain role for p53 gene alterations in human prostate cancers. *Cancer Research,* 56**,** 3814-3822.

Bubendorf, L. (1999) Survey of gene amplifications during prostate cancer progression by high throughput fluorescence in situ hybridization on tissue microarrays (vol 59, pg 803, 1999). *Cancer Research,* 59**,** 1388-1388.

Bucher, P. (1999) Regulatory elements and expression profiles. *Curr Opin Struct Biol,* 9**,** 400-7.

Buitinck, L., G. Louppe, M. Blondel, F. Pedregosa*, et al.* 2013. API design for machine learning software: experiences from the scikit-learn project. In *European Conference on Machine Learning and*

Principles and Practices of Knowledge Discovery in Databases. Prague, Czech Republic.

Cao, W. L. & F. K. Sun (2013) [PSA-based screening for prostate cancer: a comparative analysis]. *Zhonghua Nan Ke Xue,* 19**,** 559-62.

Carter, B. S., G. S. Bova, T. H. Beaty, G. D. Steinberg*, et al.* (1993) Hereditary prostate cancer: epidemiologic and clinical features. *J Urol,* 150**,** 797-802.

Carter, H. B. (2013) Prostate-specific antigen-based screening for prostate cancer: the irony of it all. *Eur Urol,* 64**,** 710-1; discussion 711-2.

Catalona, W. J., J. P. Richie, F. R. Ahmann, M. A. Hudson*, et al.* (1994) Comparison of digital rectal examination and serum prostate specific antigen in the early detection of prostate cancer: results of a multicenter clinical trial of 6,630 men. *J Urol,* 151**,** 1283-90.

Celis, J. E., T. Cabezon, J. M. A. Moreira, P. Gromov*, et al.* (2009) Molecular characterization of apocrine carcinoma of the breast: Validation of an apocrine protein signature in a well-defined cohort. *Molecular Oncology,* 3**,** 220-237.

Celis, J. E., P. Gromov, T. Cabezon, J. M. A. Moreira*, et al.* (2008) 15-Prostaglandin Dehydrogenase Expression Alone or in Combination with ACSM1 Defines a Subgroup of the Apocrine Molecular Subtype of Breast Carcinoma. *Molecular & Cellular Proteomics,* 7**,** 1795-1809.

Chang, M., K. Tsuchiya, R. H. Batchelor, P. S. Rabinovitch*, et al.* (1994) Deletion mapping of chromosome 8p in colorectal carcinoma and dysplasia arising in ulcerative colitis, prostatic carcinoma, and malignant fibrous histiocytomas. *Am J Pathol,* 144**,** 1-6.

Chen, C. D., D. S. Welsbie, C. Tran, S. H. Baek*, et al.* (2004) Molecular determinants of resistance to antiandrogen therapy. *Nature Medicine,* 10**,** 33-39.

Chen, R. B., X. Zeng, R. T. Zhang, J. T. Huang*, et al.* (2014) Ca(v)1.3 channel alpha(1D) protein is overexpressed and modulates androgen receptor transactivation in prostate cancers. *Urologic Oncology-Seminars and Original Investigations,* 32**,** 524-536.

Cheng, S. M., K. M. Fagerlund, P. Rahkila, E. Helkala*, et al.* (2007) Association of serum osteocalcin with bone gain in pubertal girls and bone loss in postmenopausal women. *Journal of Bone and Mineral Research,* 22**,** S492-S492.

Cher, M. L., G. S. Bova, D. H. Moore, E. J. Small*, et al.* (1996) Genetic alterations in untreated metastases and androgen-independent prostate cancer detected by comparative genomic hybridization and allelotyping. *Cancer Research,* 56**,** 3091-3102.

Cher, M. L., D. MacGrogan, R. Bookstein, J. A. Brown*, et al.* (1994) Comparative genomic hybridization, allelic imbalance, and fluorescence in situ hybridization on chromosome 8 in prostate cancer. *Genes Chromosomes Cancer,* 11**,** 153-62.

Chiriaco, M. S., E. Primiceri, A. Montanaro, F. de Feo*, et al.* (2013) On-chip screening for prostate cancer: an EIS microfluidic platform for contemporary detection of free and total PSA. *Analyst,* 138**,** 5404-10.

Chornokur, G., K. Dalton, M. E. Borysova & N. B. Kumar (2011) Disparities at Presentation, Diagnosis, Treatment, and Survival in African American Men, Affected by Prostate Cancer. *Prostate,* 71**,** 985-997.

Chowdhury, S., T. Dent, N. Pashayan, A. Hall*, et al.* (2013) Incorporating genomics into breast and prostate cancer screening: assessing the implications. *Genet Med,* 15**,** 423-32.

Clark-Langone, K. M., J. Y. Wu, C. Sangli, A. Chen*, et al.* (2007) Biomarker discovery for colon cancer using a 761 gene RT-PCR assay. *Bmc Genomics,* 8.

Clegg, R. M. (1992) Fluorescence Resonance Energy-Transfer and Nucleic-Acids. *Methods in Enzymology,* 211**,** 353-388.

Cooney, K. A., J. C. Wetzel, S. D. Merajver, J. A. Macoska*, et al.* (1996) Distinct regions of allelic loss on 13q in prostate cancer. *Cancer Res,* 56**,** 1142-5.

Cooperberg, M. R., J. Simko, J. Cowan, J. Reid*, et al.* (2012) Validation of a Cell-Cycle Progression Gene Panel to Improve Risk-Stratification in a Contemporary Prostatectomy Cohort. *Journal of Urology,* 187**,** E850-E850.

Cooperberg, M. R., J. P. Simko, J. E. Cowan, J. E. Reid*, et al.* (2013) Validation of a Cell-Cycle Progression Gene Panel to Improve Risk Stratification in a Contemporary Prostatectomy Cohort. *Journal of Clinical Oncology,* 31**,** 1428-1434.

Cuzick, J., D. M. Berney, G. Fisher, D. Mesher*, et al.* (2012) Prognostic value of a cell cycle progression signature for prostate cancer death in a conservatively managed needle biopsy cohort. *British Journal of Cancer,* 106**,** 1095-1099.

Cuzick, J., G. P. Swanson, G. Fisher, A. R. Brothman*, et al.* (2011) Prognostic value of an RNA expression signature derived from cell cycle proliferation genes in patients with prostate cancer: a retrospective study. *Lancet Oncology,* 12**,** 245-255.

De Coninck, V., J. Braeckman & D. Michielsen (2013) Prostate HistoScanning: a screening tool for prostate cancer? *International Journal of Urology,* 20**,** 1184-90.

de Jong, P., J. J. Catanese, K. Osoegawa, H. Shizuya*, et al.* (2001) Initial sequencing and analysis of the

human genome (vol 409, pg 860, 2001). *Nature,* 412**,** 565-566.

de Kok, J. B., R. W. Roelofs, B. A. Giesendorf, J. L. Pennings*, et al.* (2005) Normalization of gene expression measurements in tumor tissues: comparison of 13 endogenous control genes. *Laboratory Investigation,* 85**,** 154-159.

Dhanasekaran, S. M., T. R. Barrette, D. Ghosh, R. Shah*, et al.* (2001) Delineation of prognostic biomarkers in prostate cancer. *Nature,* 412**,** 822-826.

Dovey, Z., C. M. Corbishley & R. S. Kirby (2005) Prostatic intraepithelial neoplasia: a risk factor for prostate cancer. *Can J Urol,* 12 Suppl 1**,** 49-52; discussion 99-100.

Epstein, J. I. (1994) Pathology of prostatic intraepithelial neoplasia and adenocarcinoma of the prostate: prognostic influences of stage, tumor volume, grade, and margins of resection. *Seminars in Oncology,* 21**,** 527-41.

Erho, N., A. Crisan, I. A. Vergara, A. P. Mitra*, et al.* (2013) Discovery and Validation of a Prostate Cancer Genomic Classifier that Predicts Early Metastasis Following Radical Prostatectomy. *Plos One,* 8.

Felix, A. S., J. L. Weissfeld, R. M. Pfeiffer, F. Modugno*, et al.* (2014) Endometrial thickness and risk of breast and endometrial carcinomas in the prostate, lung, colorectal and ovarian cancer screening trial. *International Journal of Cancer,* 134**,** 954-60.

Feng, B., M. Srinivasan, J. R. Hoffman, J. A. Rainwater*, et al.* (2013) Physician communication regarding prostate cancer screening: analysis of unannounced standardized patient visits. *Ann Fam Med,* 11**,** 315-23.

Fodor, S. P. A., J. L. Read, M. C. Pirrung, L. Stryer*, et al.* (1991) Light-Directed, Spatially Addressable Parallel Chemical Synthesis. *Science,* 251**,** 767-773.

Freedland, S. J., L. Gerber, J. Reid, W. Welbourn*, et al.* (2013) Prognostic Utility of Cell Cycle Progression Score in Men With Prostate Cancer After Primary External Beam Radiation Therapy. *International Journal of Radiation Oncology Biology Physics,* 86**,** 848-853.

Fujiwara, Y., H. Ohata, M. Emi, K. Okui*, et al.* (1994) A 3-Mb physical map of the chromosome region 8p21.3-p22, including a 600-kb region commonly deleted in human hepatocellular carcinoma, colorectal cancer, and non-small cell lung cancer. *Genes Chromosomes Cancer,* 10**,** 7-14.

Gann, P. H. (2002) Risk factors for prostate cancer. *Rev Urol,* 4 Suppl 5**,** S3-S10.

Gaster, B. (2013) Prostate cancer screening: a complicated puzzle, explanation needed. *J Gen Intern Med,* 28**,** 1549-50.

Gaudin, P. B., I. A. Sesterhenn, K. J. Wojno, F. K. Mostofi*, et al.* (1997) Incidence and clinical significance of high-grade prostatic intraepithelial neoplasia in TURP specimens. *Urology,* 49**,** 558-63.

Gibson, U. E. M., C. A. Heid & P. M. Williams (1996) A novel method for real time quantitative RT PCR. *Genome Research,* 6**,** 995-1001.

Gleason, D. F. (1966) Classification of prostatic carcinomas. *Cancer Chemother Rep,* 50**,** 125-8.

Glinsky, G. V., A. B. Glinskii, A. J. Stephenson, R. M. Hoffman*, et al.* (2004) Gene expression profiling predicts clinical outcome of prostate cancer. *Journal of Clinical Investigation,* 113**,** 913-923.

Glinsky, G. V., A. Krones-Herzig, A. B. Glinskii & G. Gebauer (2003) Microarray analysis of xenograft-derived cancer cell lines representing multiple experimental models of human prostate cancer. *Molecular Carcinogenesis,* 37**,** 209-221.

Goeman, L., S. Joniau, D. Ponette, F. Van der Aa*, et al.* (2003) Is low-grade prostatic intraepithelial neoplasia a risk factor for cancer? *Prostate Cancer Prostatic Dis,* 6**,** 305-10.

Groskopf, J., S. M. J. Aubin, I. L. Deras, A. Blase*, et al.* (2006) APTIMA PCA3 molecular urine test: Development of a method to aid in the diagnosis of prostate cancer. *Clinical Chemistry,* 52**,** 1089-1095.

Gulati, R. & R. Etzioni (2013) Alternative prostate cancer screening strategies--in response. *Ann Intern Med,* 158**,** 778-9.

Harma, V., H. P. Schukov, A. Happonen, I. Ahonen*, et al.* (2014) Quantification of dynamic morphological drug responses in 3D organotypic cell cultures by automated image analysis. *PLoS One,* 9**,** e96426.

Heidenreich, A., P. J. Bastian, J. Bellmunt, M. Bolla*, et al.* (2014) EAU Guidelines on Prostate Cancer. Part 1: Screening, Diagnosis, and Local Treatment with Curative Intent—Update 2013. *European Urology,* 65**,** 124-137.

Henshall, S. M., D. E. H. Afar, J. Hiller, L. G. Horvath*, et al.* (2003) Survival analysis of genome-wide gene expression profiles of prostate cancers identifies new prognostic targets of disease relapse. *Cancer Research,* 63**,** 4196-4203.

Hessels, D., F. P. Smit, G. W. Verhaegh, J. A. Witjes*, et al.* (2007) Detection of TMPRSS2-ERG fusion transcripts and prostate cancer antigen 3 in urinary sediments may improve diagnosis of prostate cancer. *Clinical Cancer Research,* 13**,** 5103-5108.

Hockel, A. & N. Dornhofer (2005) The hydra phenomenon of cancer: Why tumors recur locally after microscopically complete resection. *Cancer Research,* 65**,** 2997-3002.

Hod, Y. (1992) A simplified ribonuclease protection assay. *Biotechniques,* 13**,** 852-4.

Hong, M. K. H., G. Macintyre, D. C. Wedge, P. Van Loo, *et al.* (2015) Tracking the origins and drivers of subclonal metastatic expansion in prostate cancer. *Nature Communications,* 6.

Howard, D. H. (2013) Prostate cancer screening and incidence: a question of causality--reply. *JAMA Intern Med,* 173**,** 392-3.

Howard, D. H., F. K. Tangka, G. P. Guy, D. U. Ekwueme, *et al.* (2013) Prostate cancer screening in men ages 75 and older fell by 8 percentage points after Task Force recommendation. *Health Aff (Millwood),* 32**,** 596-602.

Huggett, J., K. Dheda, S. Bustin & A. Zumla (2005) Real-time RT-PCR normalisation; strategies and considerations. *Genes and Immunity,* 6**,** 279-284.

Ibrayev, Y., K. Oda, G. E. Fraser & S. F. Knutsen (2013) Utilization of prostate cancer screening according to dietary patterns and other demographic variables. The adventist health study-2. *J Cancer,* 4**,** 416-26.

Ilic, D., M. M. Neuberger, M. Djulbegovic & P. Dahm (2013) Screening for prostate cancer. *Cochrane Database Syst Rev,* 1**,** CD004720.

Imbert, A., M. Chaffanet, L. Essioux, T. Noguchi, *et al.* (1996) Integrated map of the chromosome 8p12-p21 region, a region involved in human cancers and Werner syndrome. *Genomics,* 32**,** 29-38.

Isaacs, J. T. (1994) Role of Androgens in Prostatic-Cancer. *Vitamins and Hormones, Vol 49,* 49**,** 433-502.

Ittmann, M. (1996) Allelic loss on chromosome 10 in prostate adenocarcinoma. *Cancer Res,* 56**,** 2143-7.

Iversen, P., P. O. Madsen & D. K. Corle (1995) Radical prostatectomy versus expectant treatment for early carcinoma of the prostate. Twenty-three year follow-up of a prospective randomized study. *Scand J Urol Nephrol Suppl,* 172**,** 65-72.

Jiang, Z., B. A. Woda, K. L. Rock, Y. Xu, *et al.* (2002a) P504S: A new molecular marker for the detection of prostate carcinoma. *Journal of Urology,* 168**,** 374-374.

Jiang, Z., B. A. Woda, K. L. Rock, Y. D. Xu, *et al.* (2001) P504S - A new molecular marker for the detection of prostate carcinoma. *American Journal of Surgical Pathology,* 25**,** 1397-1404.

Jiang, Z., C. L. Wu, B. A. Woda, K. Dresser, *et al.* (2002b) P504S/alpha-methylacyl-CoA racemase - A useful marker for diagnosis of small foci of prostatic carcinoma on needle biopsy. *American Journal of Surgical Pathology,* 26**,** 1169-1174.

Joniau, S., L. Goeman, J. Pennings & H. Van Poppel (2005) Prostatic intraepithelial neoplasia (PIN): importance and clinical management. *Eur Urol,* 48**,** 379-85.

Kacprzyk, L. A., M. Laible, T. Andrasiuk, J. C. Brase, *et al.* (2013) ERG Induces Epigenetic Activation of Tudor Domain-Containing Protein 1 (TDRD1) in ERG Rearrangement-Positive Prostate Cancer. *Plos One,* 8.

Karhunen, U., L. Jaakkola, Q. Wang, U. Lamminmaki, *et al.* (2010) Luminescence switching by hybridization-directed mixed lanthanide complex formation. *Anal Chem,* 82**,** 751-4.

Karrer, E. E., J. E. Lincoln, S. Hogenhout, A. B. Bennett, *et al.* (1995) In situ isolation of mRNA from individual plant cells: creation of cell-specific cDNA libraries. *Proc Natl Acad Sci U S A,* 92**,** 3814-8.

Kilpelainen, T. P., T. L. Tammela, N. Malila, M. Hakama, *et al.* (2013) Prostate cancer mortality in the Finnish randomized screening trial. *J Natl Cancer Inst,* 105**,** 719-25.

Klein, E. A., T. Maddala, C. Millward, D. B. Cherbavaz, *et al.* (2012) Development of a needle biopsy-based genomic test to improve discrimination of clinically aggressive from indolent prostate cancer. *Journal of Clinical Oncology,* 30.

Kobayashi, T., R. Goto, S. Hinotsu & O. Ogawa (2013) [Socioeconomic advantages of longer screening intervals for men with low prostate-specific antigen levels in prostate cancer mass screening]. *Hinyokika Kiyo,* 59**,** 159-66.

Koivisto, P., J. Kononen, C. Palmberg, T. Tammela, *et al.* (1997) Androgen receptor gene amplification: A possible molecular mechanism for androgen deprivation therapy failure in prostate cancer. *Cancer Research,* 57**,** 314-319.

Konety, B., S. M. Zappala, D. J. Parekh, D. Osterhout, *et al.* (2015) The 4Kscore(R) Test Reduces Prostate Biopsy Rates in Community and Academic Urology Practices. *Rev Urol,* 17**,** 231-40.

Kristiansen, G. (2009) [Immunohistochemical algorithms in prostate diagnostics: what's new?]. *Pathologe,* 30 Suppl 2**,** 146-53.

Kumar, S., M. Shelley, C. Harrison, B. Coles, *et al.* (2006) Neo-adjuvant and adjuvant hormone therapy for localised and locally advanced prostate cancer. *Cochrane Database Syst Rev***,** CD006019.

Latil, A., I. Bieche, L. Chene, I. Laurendeau, *et al.* (2003) Gene expression profiling in clinically localized prostate cancer: A four-gene expression model predicts clinical behavior. *Clinical Cancer Research,* 9**,** 5477-5485.

Lee, H. Y. & Y. Jung (2013) Older Korean American men's prostate cancer screening behavior: the prime role of culture. *J Immigr Minor Health,* 15**,** 1030-7.

Lee, Y. J., J. E. Park, B. R. Jeon, S. M. Lee, *et al.* (2013) Is prostate-specific antigen effective for

population screening of prostate cancer? A systematic review. *Ann Lab Med,* 33**,** 233-41.

Leyten, G. H. J. M., D. Hessels, F. P. Smit, S. A. Jannink*, et al.* (2015) Identification of a Candidate Gene Panel for the Early Diagnosis of Prostate Cancer. *Clinical Cancer Research,* 21**,** 3061-3070.

Li, L., Y. Q. Du, X. Y. Kong, Z. S. Li*, et al.* (2013) Lamin B1 Is a Novel Therapeutic Target of Betulinic Acid in Pancreatic Cancer. *Clinical Cancer Research,* 19**,** 4651-4661.

Li, R. N. & Y. Shen (2013) An old method facing a new challenge: Re-visiting housekeeping proteins as internal reference control for neuroscience research. *Life Sciences,* 92**,** 747-751.

Li, Y. W., X. Hong, M. Hussain, S. H. Sarkar*, et al.* (2005) Gene expression profiling revealed novel molecular targets of docetaxel and estramustine combination treatment in prostate cancer cells. *Molecular Cancer Therapeutics,* 4**,** 389-398.

Lichtenstein, P., N. V. Holm, P. K. Verkasalo, A. Iliadou*, et al.* (2000) Environmental and heritable factors in the causation of cancer--analyses of cohorts of twins from Sweden, Denmark, and Finland. *N Engl J Med,* 343**,** 78-85.

Linja, M. J., K. P. Porkka, Z. K. Kang, K. J. Savinainen*, et al.* (2004) Expression of androgen receptor coregulators in prostate cancer. *Clinical Cancer Research,* 10**,** 1032-1040.

Lippi, G., C. Mattiuzzi & G. Cervellin (2013) Prostate-specific antigen for prostate cancer screening: a different strategy should be planned. *JAMA Intern Med,* 173**,** 392.

Lipshutz, R. J., S. P. A. Fodor, T. R. Gingeras & D. J. Lockhart (1999) High density synthetic oligonucleotide arrays. *Nature Genetics,* 21**,** 20-24.

Litovkin, K., S. Joniau, E. Lerut, A. Laenen*, et al.* (2014) Methylation of PITX2, HOXD3, RASSF1 and TDRD1 predicts biochemical recurrence in high-risk prostate cancer. *Journal of Cancer Research and Clinical Oncology,* 140**,** 1849-1861.

Loughlin, K. R. (2014) Calcium channel blockers and prostate cancer. *Urol Oncol,* 32**,** 537-8.

Lucarelli, G., M. Rutigliano, C. Bettocchi, S. Palazzo*, et al.* (2013) Spondin-2, a Secreted Extracellular Matrix Protein, is a Novel Diagnostic Biomarker for Prostate Cancer. *Journal of Urology,* 190**,** 2271-2277.

Luo, J., D. J. Duggan, Y. D. Chen, J. Sauvageot*, et al.* (2001) Human prostate cancer and benign prostatic hyperplasia: Molecular dissection by gene expression profiling. *Cancer Research,* 61**,** 4683-4688.

Luo, J., T. Dunn, C. Ewing, J. Sauvageot*, et al.* (2002a) Gene expression signature of benign prostatic hyperplasia revealed by cDNA microarray analysis. *Prostate,* 51**,** 189-200.

Luo, J., S. Zha, W. R. Gage, T. A. Dunn*, et al.* (2002b) alpha-Methylacyl-CoA racemase: A new molecular marker for prostate cancer. *Cancer Research,* 62**,** 2220-2226.

Lövgren, T., Pettersson, K. 1990. *Time-resolved fluoroimmunoassay, advantages and limitations.* Florida: CRC Press.

Macgregor, P. F. & J. A. Squire (2002) Application of microarrays to the analysis of gene expression in cancer. *Clinical Chemistry,* 48**,** 1170-1177.

Magee, J. A., T. Araki, S. Patil, T. Ehrig*, et al.* (2001) Expression profiling reveals hepsin overexpression in prostate cancer. *Cancer Research,* 61**,** 5692-5696.

Makovey, I., A. J. Stephenson & S. Haywood (2013) Response to the U.S. Preventative Services Task Force decision on prostate cancer screening. *Curr Urol Rep,* 14**,** 168-73.

Massoner, P., K. G. Kugler, K. Unterberger, R. Kuner*, et al.* (2013) Characterization of Transcriptional Changes in ERG Rearrangement-Positive Prostate Cancer Identifies the Regulation of Metabolic Sensors Such as Neuropeptide Y. *Plos One,* 8.

Matsuyama, H., Y. Pan, L. Skoog, B. Tribukait*, et al.* (1994) Deletion Mapping of Chromosome 8p in Prostate-Cancer by Fluorescence in-Situ Hybridization. *Oncogene,* 9**,** 3071-3076.

McEntee, M. F., J. I. Epstein, R. Syring, L. A. Tierney*, et al.* (1996) Characterization of prostatic basal cell hyperplasia and neoplasia in aged macaques: comparative pathology in human and nonhuman primates. *Prostate,* 29**,** 51-9.

McMenamin, M. E., P. Soung, S. Perera, I. Kaplan*, et al.* (1999) Loss of PTEN expression in paraffin-embedded primary prostate cancer correlates with high gleason score and advanced stage. *Cancer Research,* 59**,** 4291-4296.

McNeal, J. E. (1968) Regional morphology and pathology of the prostate. *Am J Clin Pathol,* 49**,** 347-57.

McNeal, J. E. (1980) Anatomy of the prostate: an historical survey of divergent views. *Prostate,* 1**,** 3-13.

McShane, L. M., D. G. Altman, W. Sauerbrei, S. E. Taube*, et al.* (2006) REporting recommendations for tumor MARKer prognostic studies (REMARK). *Breast Cancer Research and Treatment,* 100**,** 229-235.

McShane, L. M., D. G. Altman, W. Sauerbrei, S. E. Taube*, et al.* (2005) REporting recommendations for tumour MARKer prognostic studies (REMARK). *British Journal of Cancer,* 93**,** 387-391.

Melamed, J., J. M. Einhorn & M. M. Ittmann (1997) Allelic loss on chromosome 13q in human prostate carcinoma. *Clin Cancer Res,* 3, 1867-72.

Melnikow, J., M. LeFevre, T. J. Wilt & V. A. Moyer (2013) Counterpoint: Randomized trials provide the strongest evidence for clinical guidelines: The US Preventive Services Task Force and Prostate Cancer Screening. *Med Care,* 51, 301-3.

Miocinovic, R., U. Bumbasirevic, M. L. Djordjevic, N. Bojanic*, et al.* (2013) Optimal use of prostate specific antigen for prostate cancer screening. *Vojnosanit Pregl,* 70, 501-3.

Morrison, T. B., J. J. Weis & C. T. Wittwer (1998) Quantification of low-copy transcripts by continuous SYBR (R) green I monitoring during amplification. *Biotechniques,* 24, 954-+.

Moss, S. F., V. Krivosheyev, A. de Souza, K. Chin*, et al.* (1999) Decreased and aberrant nuclear lamin expression in gastrointestinal tract neoplasms. *Gut,* 45, 723-729.

Mullis, K., F. Faloona, S. Scharf, R. Saiki*, et al.* (1986) Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. *Cold Spring Harb Symp Quant Biol,* 51 Pt 1, 263-73.

Murphy, A. B., A. Macejko, A. Taylor & R. B. Nadler (2009) Chronic prostatitis: management strategies. *Drugs,* 69, 71-84.

Nakayama, M., M. L. Gonzalgo, S. Yegnasubramanian, X. H. Lin*, et al.* (2004) GSTP1 CpG island hypermethylation as a molecular biomarker for prostate cancer. *Journal of Cellular Biochemistry,* 91, 540-552.

Nurmi, J., H. Lilja & A. Ylikoski (2000a) Time-resolved fluorometry in end-point and real-time PCR quantification of nucleic acids. *Luminescence,* 15, 381-388.

Nurmi, J., A. Ylikoski, T. Soukka, M. Karp*, et al.* (2000b) A new label technology for the detection of specific polymerase chain reaction products in a closed tube. *Nucleic Acids Res,* 28, E28.

Ogden, G. R., J. G. Cowpe & M. W. Green (1990) Evidence of Field Change in Oral-Cancer. *British Journal of Oral & Maxillofacial Surgery,* 28, 390-392.

Ory, S., H. Brazier & A. Blangy (2007) Identification of a bipartite focal adhesion localization signal in RhoU/Wrch-1, a Rho family GTPase that regulates cell adhesion and migration. *Biology of the Cell,* 99, 701-716.

Ostrander, E. A., K. Markianos & J. L. Stanford (2004) Finding prostate cancer susceptibility genes. *Annu Rev Genomics Hum Genet,* 5, 151-75.

Pacelli, A. & D. G. Bostwick (1997) Clinical significance of high-grade prostatic intraepithelial neoplasia in transurethral resection specimens. *Urology,* 50, 355-9.

Parker, R. M. & N. M. Barnes (1999) mRNA: detection by in Situ and northern hybridization. *Androgen Action: Methods and Protocols,* 106, 247-83.

Pascal, L. E., R. Z. N. Vencio, L. S. Page, E. S. Liebeskind*, et al.* (2009) Gene expression relationship between prostate cancer cells of Gleason 3, 4 and normal epithelial cells as revealed by cell type-specific transcriptomes. *Bmc Cancer,* 9.

Patel, A. R., J. S. Jones, J. Rabets, G. DeOreo*, et al.* (2004) Parasagittal biopsies add minimal information in repeat saturation prostate biopsy. *Urology,* 63, 87-89.

Perner, S., J. M. Mosquera, F. Demichelis, M. D. Hofer*, et al.* (2007) TMPRSS2-ERG fusion prostate cancer: An early molecular event associated with invasion. *American Journal of Surgical Pathology,* 31, 882-888.

Powell, I. J., C. H. Bock, J. J. Ruterbusch & W. Sakr (2010) Evidence Supports a Faster Growth Rate and/or Earlier Transformation to Clinically Significant Prostate Cancer in Black Than in White American Men, and Influences Racial Progression and Mortality Disparity. *Journal of Urology,* 183, 1792-1796.

Punnen, S., N. Pavan & D. J. Parekh (2015) Finding the Wolf in Sheep's Clothing: The 4Kscore Is a Novel Blood Test That Can Accurately Identify the Risk of Aggressive Prostate Cancer. *Rev Urol,* 17, 3-13.

Qian, X. L., C. L. Li, B. Pang, M. Xue*, et al.* (2012) Spondin-2 (SPON2), a More Prostate-Cancer-Specific Diagnostic Biomarker. *Plos One,* 7.

Rappolee, D. A., D. Mark, M. J. Banda & Z. Werb (1988) Wound macrophages express TGF-alpha and other growth factors in vivo: analysis by mRNA phenotyping. *Science,* 241, 708-12.

Rhodes, D. R., T. R. Barrette, M. A. Rubin, D. Ghosh*, et al.* (2002) Meta-analysis of microarrays: Interstudy validation of gene expression profiles reveals pathway dysregulation in prostate cancer. *Cancer Research,* 62, 4427-4433.

Roobol, M. J., R. Kranse, C. H. Bangma, S. J. Otto*, et al.* (2013) Reply from authors re: Michael Baum. Screening for prostate cancer: can we learn from the mistakes of the breast screening experience? Eur Urol 2013;64:540-1: screening for prostate cancer: we have learned and are still learning. *Eur Urol,* 64, 541-3.

Rubin, M. A., M. Zhou, S. M. Dhanasekaran, S. Varambally*, et al.* (2002) alpha-methylacyl coenzyme A racemase as a tissue biomarker for prostate cancer. *Jama-Journal of the American Medical Association,* 287, 1662-1670.

Rubio-Briones, J., J. Casanova, R. Dumont, L. Rubio, *et al.* (2014) Optimizing prostate cancer screening; prospective randomized controlled study of the role of PSA and PCA3 testing in a sequential manner in an opportunistic screening program. *Actas Urol Esp,* 38**,** 217-23.

Saccomanno, C. F., M. Bordonaro, J. S. Chen & J. L. Nordstrom (1992) A faster ribonuclease protection assay. *Biotechniques,* 13**,** 846-50.

Salomon, G. (2013) Editorial comment from Dr Salomon to prostate HistoScanning: a screening tool for prostate cancer? *International Journal of Urology,* 20**,** 1191.

Sammon, J. D., S. Sukumar & Q. D. Trinh (2013) Prostate cancer screening and incidence: a question of causality. *JAMA Intern Med,* 173**,** 392-3.

Saric, T., Z. Brkanac, D. A. Troyer, S. S. Padalecki, et al. (1999) Genetic pattern of prostate cancer progression. International Journal of Cancer, 81**,** 219-224.

Sarma, A. V. & J. T. Wei (2012) Benign Prostatic Hyperplasia and Lower Urinary Tract Symptoms. New England Journal of Medicine, 367**,** 248-257.

Schadt, E. E., S. Turner & A. Kasarskis (2011) A window into third generation sequencing (vol 19, pg R227, 2010). Human Molecular Genetics, 20**,** 853-853.

Schena, M., D. Shalon, R. W. Davis & P. O. Brown (1995) Quantitative Monitoring of Gene-Expression Patterns with a Complementary-DNA Microarray. Science, 270**,** 467-470.

Schlicht, M., B. Matysiak, T. Brodzeller, X. Y. Wen, et al. (2004) Cross-species global and subset gene expression profiling identifies genes involved in prostate cancer response to selenium. Bmc Genomics, 5.

Schroder, F. H., J. Hugosson, M. J. Roobol, T. L. J. Tammela, et al. (2014) Screening and prostate cancer mortality: results of the European Randomised Study of Screening for Prostate Cancer (ERSPC) at 13 years of follow-up. Lancet, 384**,** 2027-2035.

Scott, A., R. Ambannavar, J. Jeong, M. L. Liu, et al. (2011) RT-PCR-Based Gene Expression Profiling for Cancer Biomarker Discovery from Fixed, Paraffin-Embedded Tissues. Formalin-Fixed Paraffin-Embedded Tissues: Methods and Protocols, 724**,** 239-257.

Shaikhibrahim, Z., A. Lindstrot, J. Ochsenfahrt, K. Fuchs, et al. (2013) Epigenetics-related genes in prostate cancer: Expression profile in prostate cancer tissues, androgen-sensitive and -insensitive cell lines. International Journal of Molecular Medicine, 31**,** 21-25.

Shaw, E. K., J. G. Scott & J. M. Ferrante (2013) The influence of family ties on men's prostate cancer screening, biopsy, and treatment decisions. Am J Mens Health, 7**,** 461-71.

Shen, M. M. & C. Abate-Shen (2010) Molecular genetics of prostate cancer: new prospects for old challenges. Genes Dev, 24**,** 1967-2000.

Simmons, L. A. (2013) Editorial comment from Dr Simmons to prostate HistoScanning: a screening tool for prostate cancer? International Journal of Urology, 20**,** 1192.

Simpson, D., S. Feeney, C. Boyle & A. Stitt (2000) Retinal VEGF mRNA measured by SYBR Green I fluorescence: A versatile approach to quantitative PCR. Molecular Vision, 6**,** 178-183.

Singh, D., P. G. Febbo, K. Ross, D. G. Jackson, et al. (2002) Gene expression correlates of clinical prostate cancer behavior. Cancer Cell, 1**,** 203-209.

Singh, P. B., C. M. Nicholson, N. Ragavan, R. A. Blades, et al. (2009) Risk of prostate cancer after detection of isolated high-grade prostatic intraepithelial neoplasia (HGPIN) on extended core needle biopsy: a UK hospital experience. Bmc Urology, 9**,** 3.

Slaughter, D. P., H. W. Southwick & W. Smejkal (1953) Field Cancerization in Oral Stratified Squamous Epithelium - Clinical Implications of Multicentric Origin. Cancer, 6**,** 963-968.

Sogani, P. C., W. F. Whitmore, Jr., B. S. Hilaris & M. A. Batata (1980) Experience with interstitial implantation of iodine 125 in the treatment of prostatic carcinoma. Scand J Urol Nephrol Suppl, 55**,** 205-11.

Sooriakumaran, P., P. Macanas-Pirard, G. Bucca, A. Henderson, et al. (2009) A gene expression profiling approach assessing celecoxib in a randomized controlled trial in prostate cancer. Cancer Genomics Proteomics, 6**,** 93-9.

Srigley, J. R., M. B. Amin, D. G. Bostwick, D. J. Grignon, et al. (2000) Updated protocol for the examination of specimens from patients with carcinomas of the prostate gland: a basis for checklists. Cancer Committee. Archives of Pathology & Laboratory Medicine, 124**,** 1034-9.

Stamey, T. A. (2001) Prostate cancer diagnosis using a saturation needle biopsy technique after previous negative sextant biopsies - Comment. Journal of Urology, 166**,** 91-92.

Stamey, T. A., J. A. Warrington, M. C. Caldwell, Z. X. Chen, et al. (2001) Molecular genetic profiling of gleason grade 4/5 prostate cancers compared to benign prostatic hyperplasia. Journal of Urology, 166**,** 2171-2177.

Stamey, T. A., N. Yang, A. R. Hay, J. E. Mcneal, et al. (1987) Prostate-Specific Antigen as a Serum Marker

for Adenocarcinoma of the Prostate. New England Journal of Medicine, 317, 909-916.

Stanford, J. L. & E. A. Ostrander (2001) Familial prostate cancer. Epidemiol Rev, 23, 19-23.

Stewart, C. S., B. C. Leibovich, A. L. Weaver & M. M. Lieber (2001) Prostate cancer diagnosis using a saturation needle biopsy technique after previous negative sextant biopsies. Journal of Urology, 166, 86-91.

Stewart, G. D., L. Van Neste, P. Delvenne, P. Delree, et al. (2013) Clinical Utility of an Epigenetic Assay to Detect Occult Prostate Cancer in Histopathologically Negative Biopsies: Results of the MATLOC Study. Journal of Urology, 189, 1110-1116.

Suzuki, H., D. Freije, D. R. Nusskern, K. Okami, et al. (1998) Interfocal heterogeneity of PTEN/MMAC1 gene alterations in multiple metastatic prostate cancer tissues. Cancer Research, 58, 204-209.

Taylor, K. L., R. M. Williams, K. Davis, G. Luta, et al. (2013) Decision making in prostate cancer screening using decision aids vs usual care: a randomized clinical trial. JAMA Intern Med, 173, 1704-12.

Thompson, L. C. & M. R. Pokorny (2015) Multiparametric MRI in the diagnosis of prostate cancer - a generational change. Australian Family Physician, 44, 597-602.

Trybus, T. M., A. C. Burgess, K. J. Wojno, T. W. Glover, et al. (1996) Distinct areas of allelic loss on chromosomal regions 10p and 10q in human prostate cancer. Cancer Research, 56, 2263-2267.

Tyagi, S. & F. R. Kramer (1996) Molecular beacons: Probes that fluoresce upon hybridization. Nature Biotechnology, 14, 303-308.

Vaananen, R. M., H. Lilja, A. Cronin, L. Kauko, et al. (2013) Association of transcript levels of 10 established or candidate-biomarker gene targets with cancerous versus non-cancerous prostate tissue from radical prostatectomy specimens. Clinical Biochemistry, 46, 670-674.

Vaananen, R. M., N. T. Ochoa, P. J. Bostrom, P. Taimen, et al. (2015) Altered PCA3 and TMPRSS2-ERG expression in histologically benign regions of cancerous prostates: a systematic, quantitative mRNA analysis in five prostates. Bmc Urology, 15.

Vainio, P., S. Gupta, K. Ketola, T. Mirtti, et al. (2011a) Arachidonic Acid Pathway Members PLA2G7, HPGD, EPHX2, and CYP4F8 Identified as Putative Novel Therapeutic Targets in Prostate Cancer. American Journal of Pathology, 178, 525-536.

Vainio, P., L. Lehtinen, T. Mirtti, M. Hilvo, et al. (2011b) Phospholipase PLA2G7, associated with aggressive prostate cancer, promotes prostate cancer cell migration and invasion and is inhibited by statins. Oncotarget, 2, 1176-1190.

Wang, W., X. Chen, T. Li, Y. Li, et al. (2013) Screening a phage display library for a novel FGF8b-binding peptide with anti-tumor effect on prostate cancer. Exp Cell Res, 319, 1156-64.

Varambally, S., S. M. Dhanasekaran, M. Zhou, T. R. Barrette, et al. (2002) The polycomb group protein EZH2 is involved in progression of prostate cancer. Nature, 419, 624-629.

Vasarainen, H., H. Malmi, L. Maattanen, M. Ruutu, et al. (2013) Effects of prostate cancer screening on health-related quality of life: results of the Finnish arm of the European randomized screening trial (ERSPC). Acta Oncol, 52, 1615-21.

Wazir, U., M. H. Ahmed, J. M. Bridger, A. Harvey, et al. (2013) The clinicopathological significance of lamin A/C, lamin B1 and lamin B receptor mRNA expression in human breast cancer. Cellular & Molecular Biology Letters, 18, 595-611.

Weis, J. H., S. S. Tan, B. K. Martin & C. T. Wittwer (1992) Detection of rare mRNAs via quantitative RT-PCR. Trends Genet, 8, 263-4.

Welsh, J. B., L. M. Sapinoso, A. I. Su, S. G. Kern, et al. (2001) Analysis of gene expression identifies candidate markers and pharmacological targets in prostate cancer. Cancer Research, 61, 5974-5978.

Whitcombe, D., J. Theaker, S. P. Guy, T. Brown, et al. (1999) Detection of PCR products using self-probing amplicons and fluorescence. Nature Biotechnology, 17, 804-807.

Vickers, A. J., A. M. Cronin, G. Aus, C. G. Pihl, et al. (2008) A panel of kallikrein markers can reduce unnecessary biopsy for prostate cancer: data from the European Randomized Study of Prostate Cancer Screening in Goteborg, Sweden. Bmc Medicine, 6.

Wilkes, R. & R. C. Tasker (2014) Intensive Care Treatment of Uncontrolled Status Epilepticus in Children: Systematic Literature Search of Midazolam and Anesthetic Therapies. Pediatric Critical Care Medicine, 15, 632-639.

Wilt, T. J. & M. K. Brawer (1994) The Prostate Cancer Intervention Versus Observation Trial: a randomized trial comparing radical prostatectomy versus expectant management for the treatment of clinically localized prostate cancer. J Urol, 152, 1910-4.

Vis, A. N. & T. H. Van Der Kwast (2001) Prostatic intraepithelial neoplasia and putative precursor lesions of prostate cancer: a clinical perspective. BJU Int, 88, 147-57.

Visakorpi, T., E. Hyytinen, P. Koivisto, M. Tanner, et al. (1995) In-Vivo Amplification of the Androgen Receptor Gene and Progression of Human Prostate-Cancer. Nature Genetics, 9, 401-406.

Wistuba, I. I., C. Behrens, A. K. Virmani, S. Milchgrub, et al. (1999) Allelic losses at

chromosome 8p21-23 are early and frequent events in the pathogenesis of lung cancer. Cancer Research, 59, 1973-1979.

Wittwer, C. T., M. G. Herrmann, A. A. Moss & R. P. Rasmussen (1997a) Continuous fluorescence monitoring of rapid cycle DNA amplification. Biotechniques, 22, 130-1, 134-8.

Wittwer, C. T., M. G. Herrmann, A. A. Moss & R. P. Rasmussen (1997b) Continuous fluorescence monitoring of rapid cycle DNA amplification. Biotechniques, 22, 130-&.

Voeller, H. J., M. Augustus, V. Madike, G. S. Bova, et al. (1997) Coding region of NKX3.1, a prostate-specific homeobox gene on 8p21, is not mutated in human prostate cancers. Cancer Research, 57, 4455-4459.

Woodson, K., K. J. O'Reilly, J. C. Hanson, D. Nelson, et al. (2008) The usefulness of the detection of GSTP1 methylation in urine as a biomarker in the diagnosis of prostate cancer. Journal of Urology, 179, 508-511.

Wu, C. L., B. E. Schroeder, X. J. Ma, C. J. Cutie, et al. (2013) Development and validation of a 32-gene prognostic index for prostate cancer progression. Proceedings of the National Academy of Sciences of the United States of America, 110, 6121-6126.

Xiao, Q., A. W. Hsing, Y. Park, S. C. Moore, et al. (2014) Body mass index and mortality among blacks and whites adults in the Prostate, Lung, Colorectal, and Ovarian (PLCO) cancer screening trial. Obesity (Silver Spring), 22, 260-8.

Xu, J. C., J. A. Stolk, X. Q. Zhang, S. J. Silva, et al. (2000) Identification of differentially expressed genes in human prostate cancer using subtraction and microarray. Cancer Research, 60, 1677-1682.

Zelefsky, M. J. & W. F. Whitmore, Jr. (1997) Long-term results of retropubic permanent 125iodine implantation of the prostate for clinically localized prostatic cancer. J Urol, 158, 23-9; discussion 29-30.

Zhang, J., J. Q. Xiang, Y. W. Zhang, Y. Y. Chen, et al. (2007) Cell adhesion regulates expression of the androgen receptor and coregulators in different prostate cancer cells. International Journal of Molecular Sciences, 8, 156-165.

Zhang, W., L. R. Kapusta, J. M. Slingerland & L. H. Klotz (1998) Telomerase activity in prostate cancer, prostatic intraepithelial neoplasia, and benign prostatic epithelium. Cancer Res, 58, 619-21.

Zoladz, P. R. & D. M. Diamond (2013) Current status on behavioral and biological markers of PTSD: A search for clarity in a conflicting literature. Neuroscience and Biobehavioral Reviews, 37, 860-895.

Zuzana, O., L. Ross, H. Fraser, B. Charis, et al. (2013) Screening for prostate cancer in New Zealand general practice. J Med Screen, 20, 49-51.