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University of Turku

QUANTITATIVE ANALYSIS OF NOVEL PROSTATE CANCER MARKERS IN TISSUE

Riina-Minna Väänänen

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

Professor Emeritus Timo Lövgren
Department of Biochemistry
Molecular Biotechnology and Diagnostics
University of Turku
Turku, Finland

Jussi Nurmi, Ph. D.
Department of Biochemistry
Molecular Biotechnology and Diagnostics
University of Turku
Turku, Finland
currently affiliated with the European
Patent Office, The Netherlands

Reviewed by

Outi Saramäki, Ph. D.
BioMediTech
University of Tampere
Tampere, Finland

Gerald 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

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The beginning of knowledge
is the discovery of something we do not understand.

-Frank Herbert

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, referred to in the text by their Roman numerals (**I-IV**):

I   Väänänen RM, Rissanen M, Kauko O, Junnila S, Väisänen V, Nurmi J, Alanen K, Nurmi M, Pettersson K. (2008) Quantitative real-time RT-PCR assay for *PCA3*. *Clin Biochem* **41**:103-108.

II   Väänänen RM, Lilja H, Cronin AM, Kauko L, Rissanen M, Kauko O, Kekki H, Vidbäck S, Nurmi M, Alanen K, Pettersson K. (2013) Association of transcript levels of 10 established or candidate-biomarker gene targets with cancerous versus non-cancerous prostate tissue from radical prostatectomy specimens. *Clin Biochem* **46**:670-674.

III   Väänänen RM, Lilja H, Kauko L, Helo P, Kekki H, Cronin AM, Vickers AJ, Nurmi M, Alanen K, Bjartell A, Pettersson K. (2014) Cancer-associated changes in the expression of *TMPRSS2-ERG*, *PCA3* and *SPINK1* in histologically benign tissue from cancerous vs noncancerous prostatectomy specimens. *Urology* **83**:511.e1-7.

IV   Väänänen RM, Tong Ochoa N, Boström PJ, Taimen P, Pettersson K. Systematic analysis of *PCA3* and *TMPRSS2-ERG* mRNA expression in different regions of cancerous prostate tissue. *Submitted manuscript.*

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ABBREVIATIONS

A	adenine
AUC	area under the curve
BMP-6	bone morphogenetic protein 6
bp	base pair
BPH	benign prostatic hyperplasia
C	cytosine
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
C_q	quantification cycle
CREB	cAMP response element-binding
CRPC	castration-resistant prostate cancer
DMPC	dimethylpyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide
DRE	digital rectal examination
dT	deoxythymine
ERG	ETV-related gene
FDA	Food and Drug Administration
FFPE	formalin-fixed, paraffin-embedded
FGF	fibroblast growth factor
FGF-8b	fibroblast growth factor 8, isoform b
FISH	fluorescence *in situ* hybridization
G	guanine
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GOLPH2	Golgi phosphoprotein 2
hGK-1	human glandular kallikrein 1
hK2	human kallikrein 2 (protein encoded by the *KLK2* gene)
kb	kilobase
KLK2	kallikrein-related peptidase 2 (the KLK2 protein is also known as hK2)
KLK3	kallikrein-related peptidase 3 (the KLK3 protein is also known as PSA)
KLK4	kallikrein-related peptidase 4
KLK15	kallikrein-related peptidase 15
miRNA	microRNA
MRI	magnetic resonance imaging
lncRNA	long noncoding RNA
mRNA	messenger ribonucleic acid
MSMB	beta-microseminoprotein
PCA3	prostate cancer antigen 3
PCR	polymerase chain reaction
phi	Prostate Health Index
PIN	prostatic intraepithelial neoplasia
PSA	prostate specific antigen (protein encoded by the *KLK3* gene)
PSCA	prostate stem cell antigen
qRT-PCR	quantitative reverse-transcription polymerase chain reaction
RNA	ribonucleic acid
ROC	receiver operating characteristic
RT-PCR	reverse-transcription polymerase chain reaction

siRNA	small interfering RNA
SNP	single nucleotide polymorphism
SPINK1	serine protease inhibitor, Kazal type 1
T	thymine
TATI	tumor-associated trypsin inhibitor
TEAA	triethylamine acetate
TGF	transforming growth factor
TMPRSS2	transmembrane protease, serine 2
TRPM8	transient receptor potential melastatin 8
TURP	transurethral resection of the prostate

ABSTRACT

Prostate cancer is a heterogeneous disease affecting an increasing number of men all over the world, but particularly in the countries with the Western lifestyle. The best biomarker assay currently available for the diagnosis of the disease, the measurement of prostate specific antigen (PSA) levels from blood, lacks specificity, and even when combined with invasive tests such as digital rectal exam and prostate tissue biopsies, these methods can both miss cancers, and lead to overdiagnosis and subsequent overtreatment of cancers. Moreover, they cannot provide an accurate prognosis for the disease. Due to the high prevalence of indolent prostate cancers, the majority of men affected by prostate cancer would be able to live without any medical intervention. Their latent prostate tumors would not cause any clinical symptoms during their lifetime, but few are willing to take the risk, as currently there are no methods or biomarkers to reliably differentiate the indolent cancers from the aggressive, lethal cases that really are in need of immediate medical treatment.

This doctoral work concentrated on validating 12 novel candidate genes for use as biomarkers for prostate cancer by measuring their mRNA expression levels in prostate tissue and peripheral blood of men with cancer as well as unaffected individuals. The panel of genes included the most prominent markers in the current literature: *PCA3* and the fusion gene *TMPRSS2-ERG*, in addition to *BMP-6*, *FGF-8b*, *MSMB*, *PSCA*, *SPINK1*, and *TRPM8;* and the kallikrein-related peptidase genes 2, 3, 4, and 15. Truly quantitative reverse-transcription PCR assays were developed for each of the genes for the purpose, time-resolved fluorometry was applied in the real-time detection of the amplification products, and the gene expression data were normalized by using artificial internal RNA standards.

Cancer-related, statistically significant differences in gene transcript levels were found for *TMPRSS2-ERG*, *PCA3*, and in a more modest scale, for *KLK15*, *PSCA,* and *SPINK1*. *PCA3* RNA was found in the blood of men with metastatic prostate cancer, but not in localized cases of cancer, suggesting limitations for using this method for early cancer detection in blood. *TMPRSS2-ERG* mRNA transcripts were found more frequently in cancerous than in benign prostate tissues, but they were present also in 51% of the histologically benign prostate tissues of men with prostate cancer, while being absent in specimens from men without any signs of prostate cancer. *PCA3* was shown to be 5.8 times overexpressed in cancerous tissue, but similarly to the fusion gene mRNA, its levels were upregulated also in the histologically benign regions of the tissue if the corresponding prostate was harboring carcinoma. These results indicate a possibility to utilize these molecular assays to assist in prostate cancer risk evaluation especially in men with initially histologically negative biopsies.

TIIVISTELMÄ

Eturauhassyöpä on heterogeeninen tauti, joka koskettaa kasvavaa määrää miehiä ympäri maailman, mutta erityisesti länsimaista elämäntyyliä edustavissa maissa. Eturauhassyöpädiagnostiikan tarpeisiin paras tällä hetkellä tarjolla oleva biomerkkiainemääritys, prostataspesifisen antigeenin (PSA) pitoisuuden määritys verestä, ei ole tarpeeksi spesifinen käyttötarkoitukseensa. Vaikka kyseiseen testiin yhdistetään peräsuolen kautta tehtävä eturauhasen tunnustelu ja eturauhasesta otettujen koepalojen tutkiminen kudostutkimustasolla, voi osa syövistä jäädä diagnosoimatta. Toisaalta niitä voidaan diagnosoida tarpeettomasti, mikä voi johtaa myös ylihoitamiseen. Mainitut diagnoosimenetelmät eivät myöskään pysty ennustamaan taudinkehitystä. Koska suuri osa eturauhassyövistä on hitaasti kasvavia ja kliinisesti merkityksettömiä, suurin osa miehistä, joilla syöpä todetaan, pystyisi elämään latentin kasvaimen kanssa ilman lääketieteellistä hoitoakin koko elinaikansa, ilman että kasvain ehtii aiheuttaa kliinisiä oireita missään vaiheessa. Harvat potilaat kuitenkaan valitsevat tätä vaihtoehtoa, koska käytössä ei ole menetelmiä tai biomerkkiaineita, joilla voitaisiin luotettavasti todeta, mitkä syövät kuuluvat hitaasti etenevään alaluokkaan, ja mitkä taas ovat aggressiivisia ja välitöntä lääketieteellistä hoitoa vaativia syöpiä.

Tässä väitöskirjatyössä tutkittiin kahdentoista uuden biomerkkiaineeksi ehdotetun geenin soveltuvuutta eturauhassyöpädiagnostiikkaan. Niiden lähetti-RNA-tasoja mitattiin sekä eturauhassyöpää sairastavien että terveiden henkilöiden eturauhaskudoksessa ja veressä. Tutkittuihin geeneihin kuuluivat alan kirjallisuudessa tällä hetkellä näkyvimmin esiintyvät merkkiaineet *PCA3*-geeni ja fuusiogeeni *TMPRSS2-ERG*, sekä *BMP-6-*, *FGF-8b-*, *KLK2-*, *KLK3-*, *KLK4-*, *KLK15-*, *MSMB-*, *PSCA-*, *SPINK1-* ja *TRPM8*-geenit. Kunkin geenin ilmenemistason mittaamiseen kehitettiin absoluuttisesti kvantitatiivinen käänteiskopiointi-PCR-määritys, jossa monistustuotteet havaittiin reaaliaikaisesti aikaerotteisen fluorometrian avulla, ja tulokset normalisoitiin keinotekoisen, sisäisen standardi-RNA:n avulla.

Viiden tutkitun geenin – *TMPRSS2-ERG*:n, *PCA3*:n, *KLK15*:n, *PSCA*:n ja *SPINK1*:n – ilmenemistasoissa havaittiin tilastollisesti merkitseviä, syöpään viittaavia muutoksia. *PCA3*-RNA:ta löydettiin verestä vain jo-levinneiden syöpien tapauksissa, mikä rajoittanee menetelmän käyttöä syövän varhaiseen havaitsemiseen verestä. *TMPRSS2-ERG*-lähetti-RNA:ta löydettiin useammin eturauhaskudoksesta, jos kyseessä oli syöpäkudos, mutta myös 51 prosentista niitä histologisesti hyvänlaatuisia kudoksia, jotka olivat peräisin syöpää sisältävistä eturauhasista. Tätä merkkiainetta ei kuitenkaan havaittu miehillä, joilla ei ollut todettu merkkejä eturauhassyövästä. *PCA3*-geenin osoitettiin yli-ilmenevän 5.8-kertaisesti syöpäkudoksessa, mutta sen ilmeneminen oli lisääntynyt syöpäisissä eturauhasissa myös histologisesti hyvänlaatuisilla kudosalueilla. Tulokset antavat aiheen olettaa, että molekyylitason määrityksiä voitaisiin mahdollisesti käyttää eturauhassyöpäriskin arvioinnissa erityisesti, jos potilaan ensimmäisestä eturauhaskoepalasta ei kudostasolla havaita syöpää.

1 INTRODUCTION

Prostate cancer is an increasingly common disease affecting men in all parts of the world. At an estimate, 1.1 million men were diagnosed with it worldwide in 2012, with a corresponding mortality rate of 307 000 (International Agency for Research on Cancer, 2012). These numbers deem it the second most common cancer in men after lung cancer, and the fifth leading cause of cancer-related death, accounting for 6.6% of the cancer-related deaths in men. However, mortality rates are decreasing due to the advances in cancer care and diagnostics, and not all prostate cancers are lethal by nature. Taken together, these indicate that most of the men affected by prostate cancer die rather with it than of it, and those with the indolent or slowly growing subtype could benefit more from watchful waiting management than from aggressive cancer treatments that come with the price of side effects reducing the quality of life. Yet the cases that would lead to death should be caught as early as possible, before they reach the metastatic or hormone-resistant stages at which the available treatments are only palliative. Differentiation between these two categories of disease course is, however, challenging with the current methods.

Presently, the diagnosis of prostate cancer is performed on the basis of increased prostate specific antigen (PSA) levels in serum and abnormal results in digital rectal examination (DRE) of the prostate, and confirmed by needle biopsies of the prostate tissue. The first biomarker for prostate cancer, PSA protein, was suggested for use in prostate cancer diagnostics in 1980 (Papsidero *et al.,* 1980) and its measurement from serum by immunoassays has since become a routine diagnostic tool. Despite its shortcomings, it is still the best available biomarker option for routine diagnostics of prostate cancer (Sartori & Chan 2014). The drawbacks in the sensitivity and specificity of the PSA test are that the serum PSA levels are often – but not always – elevated in cancer, and conversely, an increase in the leakage of PSA to serum can also be caused by benign conditions of the prostate. This can lead to unnecessary biopsies, and yet, the biopsy process itself can leave cancers undetected if the sampling procedure misses the carcinoma foci.

In addition to proteins, nucleic acids can serve as cancer biomarkers. In fact, during the last decade, the research efforts have resulted in reports on two novel candidate genes with high prostate cancer-specificity: *PCA3* (Bussemakers *et al.,* 1999) and *TMPRSS2-ERG* (Tomlins *et al.,* 2005). The success of *PCA3* has already been translated into a commercial urine-based test for the detection of prostate cancers with improved specificity (Groskopf *et al.,* 2006). Research on these and other novel biomarkers described in the medical literature is still ongoing, but if validated, they could potentially and conceivably be used for the early detection, prognosis, and/or monitoring of the cancer in a noninvasive manner in blood or urine, or they could complement the information obtained by histological examination of the biopsy tissue if molecular analyses are performed. However, the consensus is that no single

biomarker alone will be able to replace PSA, and instead, a combination of several markers should be used for optimal results. The ideal combination has not been found yet, and therefore studies on the cancer-related changes in the expression of the candidate genes are highly important forming the basis for the development of the new diagnostic methods for prostate cancer.

2 LITERATURE REVIEW

2.1 Prostate cancer

2.1.1 Prostate and its diseases

Prostate is a male secretory gland often described as being acorn-shaped and walnut-sized. It is located below the urinary bladder, and the urethra runs through the prostate between the bladder and the penis. The main function of the prostate is to produce fluid components for seminal fluid. During ejaculation, sperm from testes and fluid from seminal vesicles are transported through the prostate via ejaculatory ducts and mixed with the prostate-produced secretion. The alkaline seminal fluid mixture is subsequently expelled from the body through the urethra.

The prostate gland can be divided into three zones – transitional, central and peripheral zone – as suggested by McNeal (1968). The transitional zone surrounds the urethra closest to the bladder, the ejaculatory ducts run through the central zone, and the peripheral zone, containing the majority of the glandular tissue, is situated closest to the rectum. The prostate consists of muscle cells controlling the movement of the urethra, glandular cells producing the secretions, and fibrous cells forming the supportive structure of the organ. Normal prostate ductal system consists of three epithelial cell types: luminal secretory cells producing the secretory proteins; basal cells located between the luminal cells and the basement membrane; and, as the most sparse population, neuroendocrine cells supporting the growth of the luminal cells (Abate-Shen & Shen 2000).

Diseases of the prostate include inflammation, benign prostatic hyperplasia (BPH) and prostate cancer. BPH, the benign enlargement of the prostate resulting in urinary problems, is predominantly found in the transitional zone of the prostate (McNeal 1978), whereas cancers most frequently develop in the peripheral zone. Peripheral zone tumors can be palpated by a urologist through the rectum, but occult cancers that originate in the central zone can be too far inside to be reached by the DRE procedure.

2.1.2 Risk factors

The origin of prostate cancer is unknown, but several either nonmodifiable or behavioral risk factors have been reported. As reviewed by Leitzmann & Rohrmann (2012), the predetermined factors include age, ethnic origin, and a familial history of prostate cancer. In his review, Grönberg (2003) has stated that 40% of prostate cancers have a genetic component, and according to recent data, up to 25% of the familial causes of prostate cancer can be explained by the susceptibility loci in the genome (Kote-Jarai *et al.,* 2011).

Prostate cancer is predominantly seen in older men. It is diagnosed most often in men over the age of 70 with a notable increase in incidence compared to men under the age of 50 (Leitzmann & Rohrmann 2012). Fewer than 0.1% of the patients fall into the latter category (Grönberg 2003).

The incidence of prostate cancer varies depending on ethnic origin. The African-American population has been found to have a higher rate of prostate cancer than Caucasian-American men (Taksler *et al.,* 2012). Lowest incidence rates are seen in China, Japan and India (Boyle *et al.,* 1995). However, there is evidence that Asian men that do develop prostate cancer may be genetically predisposed to a more aggressive subtype (Lichtensztajn *et al.,* 2014). An increased rate has been observed in men of Japanese descent after they have immigrated to the United States, which supports the hypothesis that lifestyle factors, i.e. dietary and other behavioral habits, may have an effect on the development of the disease (Haenszel 1961).

Overall data on the dietary risk factors of prostate cancer are inconclusive, but in some studies a diet rich in meat or dairy products has been found to play a role in increasing the risk, whereas the consumption of tomatoes, soy, or cruciferous vegetables such as cabbage or broccoli would be beneficial in preventing cancer formation. Lycopene found in tomatoes is believed to protect DNA from free radicals and thereby prevent DNA damage in cells (Leitzmann & Rohrmann 2012). Isothiocyanates in cruciferous vegetables have been suggested to prevent cancers due to their ability to induce enzymes responsible for carcinogen detoxification (Joseph *et al.,* 2004).

2.1.3 Carcinogenesis

Prostate cancer is heterogeneous and multifocal by nature. Upon histological examination, the tissue can contain several distinct malignant tumor foci and precancerous lesions known as prostatic intraepithelial neoplasia (PIN). PIN can be classified as low-grade PIN or high-grade PIN which is considered as a precursor of the carcinoma. The precursor lesions are seen also in younger men, but not all will develop into clinical tumors. The development of a microscopic lesion into a clinical tumor will often take years or decades (Leitzmann & Rohrmann 2012).

In the early stages, the carcinoma is confined to the prostatic capsule. Most of these cancers are still indolent, and will not develop into a clinical disease during the lifetime of the individual. However, without clinical intervention, the prostate cancers prone to be more aggressive will later advance by invading the seminal vesicles, metastasizing with preference to the bone, and subsequently shifting to an androgen-independent stage that is now referred to as castration-resistant prostate cancer (CRPC). This results from the androgen-deprivation therapy commonly used for treatment of prostate cancer, and five different mechanisms of development are described in the review by Feldman & Feldman (2001). In the hypersensitive pathway, even reduced androgen levels can activate the androgen receptor either due to the increased sensitivity of the

receptor, increased conversion of testosterone to the more potent androgen, dihydrotestosterone, or gene amplification leading to higher amounts of the receptor itself. The stem cell hypothesis comprises the theory that a small population of epithelial stem cells that are already able to bypass the androgen regulation exists from the beginning, and depriving the cells of androgen facilitates the selective growth of those cell populations in the tumor. Mutations in the androgen receptor can lead to promiscuous activation of the receptor by antagonists such as flutamide or corticosteroids. In the bypass pathway the cell is stimulated to proliferate by unrelated pathways, and in the outlaw pathway the receptor is phosphorylated by other kinases instead of using androgen as the ligand (Feldman & Feldman 2001). De novo androgen synthesis in the tumor can drive the growth as well (Locke *et al*., 2008). The metastatic and hormone-resistant stages resulting from these developments are predominantly lethal (Abate-Shen & Shen 2000).

Prostate cancer, like all cancers, is ultimately caused by a series of subsequent molecular alterations that disrupt the control of cell cycle and/or apoptosis and lead to uncontrolled growth and proliferation of the cells. As reviewed by Mazaris and Tsiotras (2013), molecular events suggested to contribute to prostate carcinogenesis include inactivation of tumor suppressor genes such as *Rb, TP53,* or *PTEN*, or activation of oncogenes such as the gene coding for androgen receptor, leading to abnormally active, sensitive, or specific androgen signaling and thereby proliferation of prostate cells. Epigenetic changes such as mutations in genes affecting chromatin modifications can also play a role in inappropriate activation or inactivation of genes in prostate cancer (Barbieri *et al*., 2013). In general, altered cell-cycle control is thought to be the primary cause of the cancer progression at the localized stage, whereas deregulated apoptosis accounts for the growth at the further advanced phase of the cancer (Abate-Shen & Shen 2000).

2.1.4   Cancer management

When the prostate carcinoma is still confined within the capsule, the cancers are essentially curable. Treatment costs are also lower for the early-stage disease than for the advanced forms. The age and general health of the patient, the clinical stage of the cancer, and the histological grade play a role in treatment decisions (Catalona 1994).

The clinical stage describes the extent of the cancer. The so-called TNM classification reports the extent of the primary tumor (T), the status of spreading to lymph nodes (N), and the presence of metastases (M). The detailed classification system is presented in Table 1.

The most commonly used system for histological grading is the scale originally presented by Donald Gleason in 1974 (Gleason & Mellinger 1974). In the Gleason system, the tissue to be graded falls into one of five histological patterns (Gleason grades 1–5), numbered according to their increasing biologic malignancy, and grade 1

representing the most differentiated and 5 the least differentiated cell type. Gleason score is determined by adding together the two most predominant grades present in the tumor, thereby resulting in a number on a scale from 2 to 10.

Table 1. Tumor-Node-Metastasis classification of prostate cancer. Adapted from Catalona (1994) and American Joint Committee on Cancer (2014).

Category to be assessed	Stage		Definition
Primary tumor	TX		Primary tumor cannot be assessed
	T0		No evidence of tumor
	T1		Tumor clinically unapparent, not palpable or visible by imaging
		T1a	Incidental histological finding in 5% or less of the resected tissue
		T1b	Incidental histological finding in more than 5% of the resected tissue
		T1c	Identified by needle biopsy but not palpable or visible by imaging
	T2		Tumor confined within the prostate
		T2a	Involves half a lobe or less
		T2b	Involves more than half a lobe but not both lobes
		T2c	Involves both lobes
	T3		Tumor extends through the prostate capsule
		T3a	Extends unilaterally or bilaterally
		T3b	Invades seminal vesicles
	T4		Tumor fixed or invades adjacent structures other than seminal vesicles
Regional lymph nodes	NX		Lymph nodes cannot be assessed
	N0		No evidence of spread to lymph nodes
	N1		Metastasis in regional lymph nodes
Distant metastasis	MX		Metastases cannot be assessed
	M0		No distant metastases
	M1		Distant metastases
		M1a	Metastases in nonregional lymph nodes
		M1b	Metastases in bone
		M1c	Metastases at other sites

The management options for prostate cancer include active surveillance, curative treatment, and palliative care. Clinically localized disease is managed by active surveillance, if the patient has T1a cancer and a life expectancy of less than 10 years, and radical prostatectomy or radiation therapy at other T1 and T2 stages. Surgical removals of the prostate have fewer complications if performed at the early stages. Radiation therapy is chosen especially for those men who are older, and in poorer general health with clinically advanced disease and tumors of higher grade. Cancers that are locally advanced to stage T3, i.e., extended through the prostate capsule, are also treated with surgery or radiation therapy, but these may not be able to cure the disease as many men with T3 tumors also have occult metastases. Fewer than 15% of the cancers that have metastasized to lymph nodes are curable with any of the treatment modalities. The options, however, include hormonal therapy in addition to the previously mentioned surgery and radiation therapy. As the growth of prostate tumors is androgen-dependent, hormonal therapy will slow disease progression, but will not cure the cancer. The mechanisms of the hormonal therapy include suppressing gonadotropin secretion, and thereby testosterone secretion, by agonists, and blocking the binding of androgens to their receptors, among others. The symptoms of cancers that have spread beyond the lymph nodes can be palliated with hormonal therapy (Catalona 1994). However, the androgen-ablation therapy results in the recurrence of highly aggressive metastatic cancer that has escaped androgen control, as has been described in Chapter 2.1.3. Patients with the hormone-resistant cancer can be administered secondary hormonal treatments or palliative radiation therapy in order to diminish the size of the metastases, but the results are poor and temporary, and patients will relapse (Catalona 1994).

2.1.5   Diagnosis

The most prominent side effects of the treatments of prostate cancer include blood loss, impotence, and urinary incontinence (Catalona 1994). As mentioned in Chapter 2.1.3, most prostate cancers are of the indolent type, and active surveillance would suffice and be preferential for their management because, in these cases, the benefits achieved by the treatments may not outweigh the side effects. Therefore, in order to avoid the overtreatment of the clinically insignificant cancers, the ongoing challenge for the diagnostics is not only to detect the cancers early, but also to identify the men suffering from the aggressive form that requires robust intervention and treatment.

In the United States, the incidence of prostate cancer showed a sharp increase in the early 1990s (Tiwari & Roy 2012). However, the mortality rates have been fairly stable since the 1970s. The sudden increase in the incidence was largely due to improved diagnostics, as shown by the introduction of the PSA test. The current routine diagnostics involve the initial suspicion of cancer after elevated serum PSA levels and abnormal DRE results. The suspicions are confirmed or rejected by the histological examination of core needle tissue biopsies taken from the prostate through the rectum with the guidance of a transrectal ultrasound (TRUS) probe. The process is not perfect:

despite being the best-performing biomarker at the moment, PSA does not provide the sensitivity or specificity (Table 2) required for optimal diagnosis and prognosis. Elevated PSA levels can be associated with benign hyperplastic conditions (Catalona 1994), and conversely, almost a quarter of prostate cancer patients can present serum PSA levels lower than the often recommended cut-off of 4 ng/mL (Catalona *et al.,* 1997). The biopsy procedure usually consists of the examination of 6–12 biopsy cores, but it can still miss cancers, and it has been shown that simply increasing the number of tissue cores taken does not improve the detection rate (Andriole 2009, Delongchamps *et al.,* 2009). In addition to missing cancers, undergrading and understaging of tumors based on biopsies is an issue that can lead to incorrect treatment choices. Underestimations can occur due to preoperative clinical factors such as serum PSA level, or when the prostate is large or the tumor volume is not appropriately presented in the biopsies. New biomarkers to either replace or work in collaboration with PSA are therefore urgently needed – not only to aid in diagnosing the malignancy, but, ideally, also to provide more specific information on the prognosis.

Table 2. Diagnostic measures: sensitivity and specificity.

	Disease present	**Disease absent**
Test positive	True positives	False positives
Test negative	False negatives	True negatives
	↓	↓

	Sensitivity:	**Specificity:**
	$\dfrac{\text{True positives}}{\text{True positives + False negatives}}$	$\dfrac{\text{True negatives}}{\text{True negatives + False positives}}$

2.2   Finding biomarkers for prostate cancer: challenges in gene expression experiments

Research on gene expression is one of the keys to both understanding the biology of cancer, and finding suitable molecules to be used as diagnostic and prognostic biomarkers of the disease. Differential gene expression in malignant conditions can be caused by changes in the genome, such as variability in copy numbers of the genes or rearrangements of the genetic material. In addition, epigenetic changes can also regulate the expression, as well as specific clearing of mRNAs before they are used for protein translation. Studying the cell mRNA content facilitates the identification of the genes expressed, as well as the extent thereof, at the time of the sample collection. Performing the experiments in different disease and nondisease conditions allows the

identification of the genes that are important to the formation of the malignant state or, alternately, the recognition of the genes the expression of which is affected by the malignancy. Gene transcripts falling into either category could subsequently be used as biomarkers.

As molecules highly susceptible to degradation, mRNAs are a challenging group of analytes to detect: sensitive, state-of-the-art methods for sample collection, processing, and analysis are called for, but standardized protocols for these are yet to be achieved. This doctoral work concentrated on differentially expressed mRNAs in prostate tissue and this chapter of the literature review provides a background for the technological aspects of this type of gene expression research and discusses the most prevalent challenges in the experimental settings.

2.2.1 Specimen collection

Prostate cancer originates in prostate tissue, and therefore the basic understanding of gene expression alterations in neoplastic conditions starts at tissue level research. Prostate tissue material can be collected from preoperative biopsies, prostate chips produced by a transurethral resection of the prostate (TURP), prostatectomy specimens, autopsy specimens or cystoprostatectomy specimens. For molecular research, it would be important to know the exact morphology of the original tissue under study, but often this information is impossible to obtain. As is discussed in more detail in Chapter 2.2.2, the fixation of the tissue for histological examinations renders the cells inapplicable for mRNA measurements. Therefore, the surrounding adjacent tissue usually has to serve as a surrogate for the estimation of the content of the sample intended for molecular assays. In addition to cancerous tissue of different stages and grades, controls are invaluable for research set-ups. BPH tissue is often used as a control for noncancerous tissue but, as benign hyperplasia is most prevalent in the transitional zone of the prostate, whereas cancer more often develops in the peripheral zone (McNeal 1968), this may create a liability for comparisons, as genes have been found to present zone-specific expression (van der Heul-Nieuwenhuijsen *et al.,* 2006). Histologically benign tissues can be collected from cancerous prostates, but recent reports on molecular changes observed also in the regions surrounding malignant tumors (Nonn *et al.,* 2009) could conceivably diminish their value as control tissue, when differences between healthy and cancerous states are examined. Yet, due to the invasive nature of tissue collection, obtaining prostate tissue from healthy men without any prostatic diseases is challenging. Autopsy tissue from organ donors (Chan *et al.,* 1999), or prostate tissue from cystoprostatectomy specimens performed on bladder cancer patients can be an alternative source of noncancerous tissue. However, as prostate cancer is highly prevalent in the population, especially in the aging subgroup of men, microscopic tumor foci can be found even in men without clinical symptoms and, therefore, careful pathological evaluation of the prostates obtained from organ donors or bladder cancer patients should be performed to ensure that the prostates are truly free of malignant tumors. The incidental carcinoma foci are not as prevalent an

issue with organ donation specimens due to the fact that they are usually obtained from fairly young individuals.

Going towards improved diagnostics and monitoring of prostate cancer, less invasive settings would be preferable, and the use of bodily fluids such as peripheral blood and urine has been investigated. In addition to measurements with the more traditional protein assays, blood can be examined for circulating tumor cells or free circulating nucleic acids. In some types of leukemia, the detection of tumor-specific transcripts in blood to monitor the disease is already in routine clinical use (Zhen & Wang 2013). However, the high background count of millions of lymphocytes per circulating tumor cell can pose a problem in epithelial cancers such as the prostate cancer, and highly sensitive detection methods are therefore called for. Furthermore, the enrichment of tumor cells is usually required for improved sensitivity, and it can be performed by immunoaffinity techniques or other methods that specifically capture the circulating tumor cells based on their physical properties (Alix-Panabieres & Pantel 2013).

Urine has been proven to contain not only prostatic cells, shed from the prostate particularly in cancer cases, and after the manipulation of the prostate through prostatic massage or digital rectal examination; but also exosomes, vesicles of prostatic origin that can contain tissue-specific proteins; and nucleic acids. Cell-based assays for the detection of prostate cancer markers have been developed and proven to have clinical value (Groskopf *et al.,* 2006; Laxman *et al.,* 2006). Exosomes are a more recent, promising alternative target in urine-based assays (Nilsson *et al.,* 2009; Dijkstra *et al.,* 2014). They are secreted by both normal and tumor cells, and as they lack the ribosomal RNA that usually makes up the bulk of RNA in cells, they can be inherently enriched in regard to the mRNA fraction. The challenges caused by the heterogenous nature of prostate cancer in tissue sampling settings could also be overcome by examining the exosomes that could conceivably present a more uniform image of the overall tumor load in the organ (Skog *et al.,* 2008).

2.2.2   Specimen handling

Obtaining accurate data on mRNA levels in biological matrices relies on high-quality specimens, and appropriate sample collection and handling is crucial. Without stabilizing procedures, the RNA content of cells is quickly degraded by RNAses of inherent and external origins after the cells are removed from their original environment. Optimal preservation of RNA in excised samples can be achieved by snap-freezing the tissue or cell sample immediately after removal while it is still fresh, but, unfortunately, this is not always feasible (Srinivasan *et al.,* 2002). Another, more practical option is to immerse the sample in a fixative. Guanidine thiocyanate can be used to deactivate RNAses and denature the proteins, while also enabling subsequent extraction of intact RNA from samples (Chirgwin *et al.,* 1979). Specific reagent mixtures designed to stabilize RNA are also commercially available. After storage in RNAlater® (Ambion), an aqueous solution that permeates the tissue, the extracted

RNA is of equal quality to RNA extracted from snap-frozen samples (Grotzer *et al.,* 2000; Florell *et al.,* 2001). RNA in peripheral blood can be stabilized by PAXgene solution (Preanalytix) (Rainen *et al.,* 2002).

For prospective studies, the specimen collection method can be designed to fit the experimental protocol and aim. However, archived tissue material could be highly useful for retrospective studies regarding, for example, patient outcomes after several years, or later recurrences of the cancer. The tissue fixative most commonly used for histopathological purposes is formalin. Rupp and von Weisäcker and their colleagues were the first to detect any RNA from formalin-fixed samples by Northern hybridization and reverse-transcription polymerase chain reaction (RT-PCR), respectively (Rupp & Locker 1988; von Weizsacker *et al.,* 1991). Since then, the reports on the levels of success have varied, and formalin fixation is widely known to affect the RNA quality. The routine protocol of archiving the formalin-fixed tissue embedded in paraffin (FFPE) also reduces the quantity and quality of RNA if archiving is prolonged (Ibusuki *et al.,* 2013; Nam *et al.,* 2014; Granato *et al.,* 2014), and changes in RNA quality have appeared already after one month of storage (Nam *et al.,* 2014). Thus, the possibilities for detection of RNA in FFPE samples are limited and only small RNA fragments can be amplified from FFPE tissue (Srinivasan *et al.,* 2002). Amplicons of 148–200 bp in length are already long enough to be affected, and, in general, amplicons of 60–120 bp are recommended for robust analysis (Lewis *et al.,* 2001; Nam *et al.,* 2014).

The formalin fixation has been speculated to affect the RNA either by degrading it (Bresters *et al.,* 1994); causing it to be cross-linked to proteins, thus rendering the RNA poorly extractable; or otherwise chemically modifying it. There are reports showing that fragmentation before fixation and during prolonged storage in paraffin blocks at room temperature can occur, but that even seemingly intact RNA can perform poorly in RT-PCR applications: this suggests that degradation accounts for only a portion of the decrease in quality (Masuda *et al.,* 1999; von Ahlfen *et al.,* 2007). Cross-linking to proteins can be reversed by treatment with proteinase K, but the greatest challenge is the chemical modification by formalin (Masuda *et al.,* 1999). In the modification reaction, formaldehyde is attached to a nucleotide base in RNA in the form of monomethylol and, subsequently, the monomethylol forms a methylene bridge to another base by an electrophilic attack. Vulnerability to the modification has been found to vary for the nucleic acid bases: adenine was found to be the most and uracil the least easily affected. For reverse transcription, it is therefore recommended that random priming rather than oligo-dT primers be used, if the poly(A) tails of mRNA are suspected to be damaged. The methylol addition can be reversed by heating the pure RNA extract in a nonformalin buffer, such as TE buffer, prior to reverse transcription. This revives a large portion of the RNA making it an adequate template for reverse transcription. Not all modifications are reversible, however, and fixation methods other than formalin have later been suggested for fixing the tissue while simultaneously preserving nucleic acids. The alternative methods include Methacarn (consisting of

60% methanol, 30% chloroform, and 10% glacial acetic acid), RCL2® (Alphelys), Carney's fixative (consisting of 60% ethanol, 30% chloroform, and 10% glacial acetic acid), and the HOPE method (Hepes-glutamic acid buffer-mediated organic solvent protection effect) (Srinivasan *et al.,* 2002; Delfour *et al.,* 2006; Nam *et al.,* 2014). The recently developed PAXgene tissue system (Preanalytix) has also been tested in comparison to formalin fixation: it was possible to successfully amplify longer amplicons with the PAXgene system, but its ability to replace formalin is still under evaluation (Belloni *et al.,* 2013).

As none of the alternative methods have yet become common and, therefore, tissue preserved using them is not widely available for retrospective studies, researchers have used other approaches to be able to still utilize the existing, vast FFPE tissue archives. Experimental protocols have been modified to include RNA extraction kits that have been manufacturer-optimized for the reversal of formaldehyde effects, and short amplicons have been designed to maximize the possibility of an intact target region of RNA (Walter *et al.,* 2013; Gruber *et al.,* 2014). To ensure data reliability, specific criteria for nucleic acid quality have been used to exclude samples with inadequate quality (Korlimarla *et al.,* 2014).

2.2.3   Accurate quantification of mRNA transcripts

To ensure the identification of the relevant aberrations in gene expression in disease conditions, reliable quantitative data on the transcript levels are required. As reviewed by Bustin (2000), there are five techniques in use for the quantification of mRNA transcript levels: Northern blotting; *in situ* hybridization; RNA protection assays; complementary DNA (cDNA) arrays; and the most widely used, RT-PCR. However, the first three are limited in their ability to produce quantitative data, and nucleic acid amplification techniques are required for sensitive, yet quantitative analyses. Polymerase chain reaction (PCR), developed by Kary Mullis and first reported in 1985 (Saiki *et al.,* 1985), is the most widely used amplification method for nucleic acids and it is able to produce multiple copies from one or several DNA fragments in a thermocyclic reaction. Coupling the technique with reverse transcriptase enzyme retrieved from retroviruses enables also the amplification of RNA (Rappolee *et al.,* 1988). The invention of reporter systems based on detection chemistries involving fluorescent probes enabled researchers to evade the problems in end-point measurements related to the variable linearity of amplification, and produce accurate data on the amounts of amplification products in real-time. Oligonucleotide probes coupled to fluorescent labels – Molecular beacons (Tyagi & Kramer 1996), hybridization probes (Wittwer *et al.,* 1997; Karhunen *et al.,* 2011), or hydrolysis probes (Livak *et al.,* 1995; Nurmi *et al.,* 2002) – allow for the sensitive and specific detection of the amplification products, whereas the SYBR® Green technique (Morrison *et al.,* 1998) requires an additional step, a melting curve analysis for specificity.

However, to obtain a true idea of the gene expression in the specimen at the time of specimen collection, the target-specific transcript level data need to be normalized and corrected for potential RNA degradation and other variables in the multistep assay protocol. Relative quantification is widely used and takes advantage of reference genes, previously referred to as house-keeping genes. They are assumed to be transcribed at a fixed level in all conditions and in this approach, the target gene expression levels are reported as ratios to the expression level of the reference gene. Beta-actin and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) are two of the most commonly used reference genes (Bustin 2000). The reliability of this normalization method has often been questioned due to the reported changes in the expression of the reference genes in disease conditions, and their instability in FFPE tissues during storage (Tramm *et al.,* 2013; Li & Shen 2013). Therefore, the validation of the chosen reference genes for the desired application is highly essential, and the use of more than one gene is recommended. An alternative, potentially excellent method for internal reference is the use of artificial RNA that cannot be found in the specimens inherently (Nurmi *et al.,* 2000b; Huggett *et al.,* 2005). An addition of a fixed amount of the internal reference RNA to the sample before RNA extraction process allows the later detection of the internal RNA by RT-PCR, and subsequent normalization of the target mRNA level to the internal reference level. An advantage is that the standard level does not change according to sample characteristics, such as neoplasticity of the cells. However, the production of artificial RNA is an extra step of labor for the research laboratories and, furthermore, even this internal reference cannot control the changes in the sample before the addition of the reference, i.e. during sample collection and storage. In the absence of a reliable, trustworthy internal reference, research groups have sometimes opted to use total RNA concentration as the tool for normalization (Bustin 2000). This is not entirely without pitfalls either, as highly proliferating cells can produce large amounts of RNA, the bulk of which is composed of ribosomal RNA and the amount of ribosomal RNA can be affected by biological factors.

2.3   Finding biomarkers for prostate cancer: suggested mRNA transcripts

Proteomics and genomic microarray studies have increasingly provided the researchers with new candidate genes to examine more closely for potential usefulness in cancer diagnostics. The expression of the genes can be measured on translational level by detecting proteins, or on transcriptional level by examining mRNA transcripts. Proteins can quickly become degraded or they may be ineffectively translated and, therefore, an increase in mRNA level may not lead to higher protein levels, and high protein expression may not occur simultaneously with high mRNA levels. Consequently, the observations of a specific gene can vary based on the target molecule. This chapter discusses the hitherto reported mRNA transcript level data on the target genes that were examined in this doctoral work. The target mRNAs include a non-coding RNA, a fusion gene, and genes coding for proteins that act in roles as diverse as secreted

proteases, cell membrane components, and growth factors. The 12 target genes are presented in alphabetical order.

2.3.1   BMP-6 (bone morphogenetic protein 6)

Prostate cancer metastasizes predominantly to the bone. Bone morphogenetic protein 6 (*BMP-6*) is a gene that codes for a protein acting as a factor in bone tissue formation (Wozney 1989). It belongs to the TGF (transforming growth factor) -beta superfamily of growth factors (Celeste *et al.,* 1990), and normally plays a role in mesechymal differentiation initiating the osteoblastic differentiation of pluripotent precursor cells (Gitelman *et al.,* 1995). The *BMP-6* gene is located in chromosome 6p23-p24 (Hahn *et al*., 1992; Olavesen *et al*., 1997).

Using end-point RT-PCR, *BMP-6* mRNA was found to be expressed in the prostate tissue of 32% of the 19 men with prostatic adenocarcinoma, but not in the 12 studied BPH tissues or 10 controls with ocular melanoma (Bentley *et al.,* 1992). The results were later repeated with *in situ* hybridization (Hamdy *et al.,* 1997). However, there are also reports on *BMP-6* mRNA present in normal prostate tissue but at lower expression levels than in cancerous regions (Harris *et al.,* 1994; Barnes *et al.,* 1995). The relationship of *BMP-6* mRNA transcripts and prostate cancer has not been extensively studied, but its potential role in metastatic cancer has garnered particular interest. In the early study by Bentley and colleagues, *BMP-6* was the only one of the several studied *BMP* genes that had a detectable mRNA expression exclusively in patients with skeletal metastases. Of the cohort of 19 patients mentioned above, 11 were positive for metastases in bone scans and 55% of the 11 expressed *BMP-6* mRNA (Bentley *et al.,* 1992). Another, more recent report also showed that *BMP-6* mRNA was found in metastatic cancers at a higher frequency compared to localized diseases (Hamdy *et al.,* 1997).

Higher levels of circulating *BMP-6* mRNA were found in the post-treatment plasma of patients with metastases, than in patients with a locally confined disease, but a similar trend was not seen in the blood cells of the same patients (Deligezer *et al.,* 2010). Lately, the research interests have concentrated on the effect of *BMP-6* on cell proliferation rather than clinical studies regarding prostate cancer.

2.3.2   FGF-8b (fibroblast growth factor 8, isoform b)

Growth factors are signaling molecules needed for the cell growth and proliferation. *FGF-8* (fibroblast growth factor 8) gene is situated in chromosome 10q24 and codes for a protein belonging to the FGF superfamily of growth factors (Yoshiura *et al.,* 1997). FGF-8 protein functions as a mitogenic embryonic epithelial factor, and in adults it is expressed predominantly in testes and ovaries (Payson *et al.,* 1996). The physiological levels of *FGF-8* products in cells are thought to be low in general, as in contrast to the more sensitive RT-PCR method, the less sensitive technique of Northern

blotting was not able to detect expression in adult prostate, kidneys or testes (Ghosh *et al.,* 1996).

FGF-8 was previously known as AIGF, androgen-induced growth factor, and it has been shown to be capable of inducing the growth of cancerous mammary cell lines of both human and mouse origin if androgens are supplied (Tanaka *et al.,* 1992; Payson *et al.,* 1996). However, androgen-independent control mechanisms of FGF-8 expression may also exist, because some studies failed to show a regulating role for androgen in the FGF-8 expression (Schmitt *et al.,* 1996), even in the lymph node-derived prostate carcinoma cell line LNCaP that is androgen-driven (Ghosh *et al.,* 1996). Additionally, DU145 and PC3 cell lines, derived from brain and bone metastases, respectively, lack the androgen receptor, but were found to be positive for *FGF-8* mRNA (Ghosh *et al.,* 1996; Schmitt *et al.,* 1996).

Dorkin and colleagues (1999) found by *in situ* hybridization that *FGF-8* mRNA was present in 80% of the studied 106 prostate cancer cases, and that the increased expression was associated with a higher Gleason score and an advanced tumor stage. Leung and colleagues (1996) also found a correlation between high *FGF-8* mRNA levels and a poor differentiation of cells. In contrast, Wang and colleagues (1999) found *FGF-8* mRNA expression in all 14 BPH samples and in 87% of the 39 tumor samples, and indicated a negative correlation between *FGF-8* mRNA expression and the Gleason grade (Wang *et al.,* 1999). The initial expression detected by *in situ* hybridization was confirmed in a subgroup of samples with RT-PCR.

The human *FGF-8* gene consists of six exons. The identification of the four mRNA variants – a, b, e, and f – and seven protein products (Crossley & Martin 1995; MacArthur *et al.,* 1995b; Ghosh *et al.,* 1996) resulting from an alternative splicing of the first exon has enabled more targeted experiments, and it has led to the discovery that particularly the isoform b is implicated in prostate cancer. While FGF-8b protein has the strongest tumorigenic and transforming abilities of the isoforms (Ghosh *et al.,* 1996; MacArthur *et al.,* 1995a), *FGF-8b* expression has rarely, if ever, been measured at mRNA level in any clinical cohorts relating to prostate cancer.

2.3.3   Kallikrein-related peptidases 2, 3, 4, and 15

The human tissue kallikrein gene family consists of 15 members and they are situated in tandem in the chromosomal locus 19q13.4 as depicted in Figure 1 (Yousef & Diamandis 2001). It has been shown that the serine proteases encoded by these genes are able to degrade extracellular matrix components as well as produce mitogenic factors, and are, therefore, able to facilitate the growth of tumors in addition to their normal functions (Obiezu & Diamandis 2005). They all consist of five exons interspersed with four introns, and contain a signal peptide encoded within the first exon (Bhoola *et al.,* 1992). Of the 15 kallikrein genes, four – *KLK2*, *KLK3*, *KLK4*, and

KLK15 – were studied in this doctoral work, and therefore prior research especially concentrating on them is discussed here.

The expression of *KLK2* and *KLK3* is mostly restricted to prostate, while *KLK4* and *KLK15* are more widely expressed among human tissues and body fluids (Shaw & Diamandis 2007). As reviewed by Lawrence and colleagues, especially *KLK2* and *KLK3* are upregulated by androgen in the prostate, and preliminary data existing on *KLK4* and *KLK15* suggest that they are also under androgen regulation. The expression in prostate is highest for *KLK2* and *KLK3,* with *KLK4* and *KLK15* exhibiting lower levels of expression (Lawrence *et al.,* 2010; Obiezu *et al.,* 2002). Single nucleotide polymorphisms (SNPs) located within *KLK2*, *KLK3*, *KLK4,* and *KLK15* have been identified and associated with prostate cancer (Pal *et al.,* 2007; Gallagher *et al.,* 2010; Batra *et al.,* 2011; Lose *et al.,* 2012). The SNPs have been demonstrated to correlate with for example decreased risk of prostate cancer, biochemical relapse or disease-specific survival.
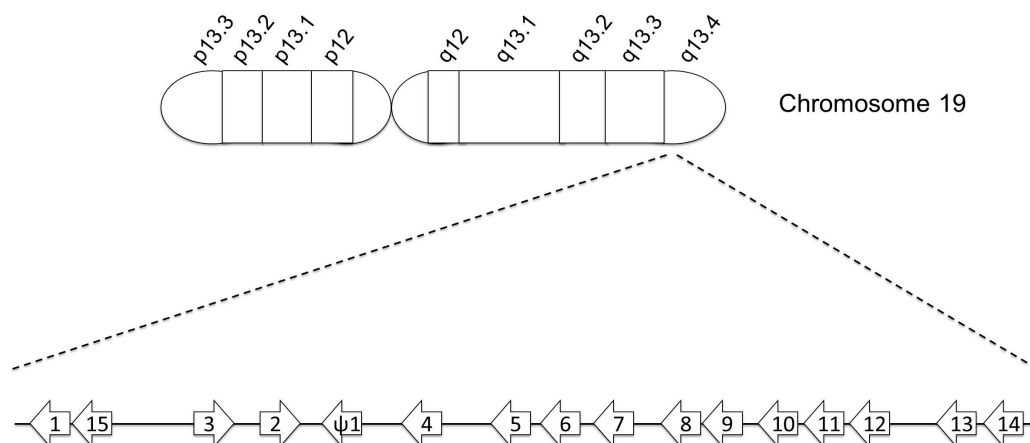
Figure 1. The kallikrein locus in chromosome 19q13.4. The locus spans approximately 300 kb and consists of the 15 kallikrein genes and one pseudogene (ψ1). The arrows indicate the direction of gene transcription. Figure modified from (Borgono & Diamandis 2004).

2.3.3.1 KLK2 (kallikrein-related peptidase 2)

For the *KLK2* gene, earlier known as human glandular kallikrein-1 (hGK-1), seven mRNA variants have been described (Kurlender *et al.,* 2005). *KLK2* expression has been detected in breast and other tissues, but it is predominantly expressed in prostate (Lawrence *et al.,* 2010). The biological function of KLK2 includes ensuring sperm motility after ejaculation through the exertion of its protease activity on the proteins responsible for clotting the seminal matter (Deperthes *et al.,* 1996). The KLK2 protein (previously also known as hK2, or human kallikrein 2), has been shown to have value

in prostate cancer diagnosis as a part of a multi-kallikrein panel (Vickers *et al.,* 2008; Vickers *et al.,* 2010).

KLK2 is claimed to be upregulated at the transcriptional level in cancerous prostate tissue in comparison to BPH tissue. This has been shown by semiquantitative RT-PCR (Neves *et al.,* 2008) and *in situ* hybridization (Herrala *et al.,* 2001). *In situ* hybridization studies and Northern blotting analyses comparing *KLK2* and *KLK3* mRNA levels in prostate tissue have shown that the *KLK2* expression is about two to ten times lower than that of *KLK3* (Chapdelaine *et al.,* 1988; Henttu *et al.,* 1990; Young *et al.,* 1992). Lintula and colleagues (2005) showed by RT-PCR that the *KLK2*/*KLK3* mRNA ratio increases in cancer tissue, and in the light of *KLK3* mRNA expression remaining fairly constant through neoplastic development as described in Chapter 2.3.3.2, these data, also, imply an increase in the *KLK2* expression in cancer.

In a study examining the ability of *KLK2* mRNA transcripts to serve as surrogates for prostate cells in the peripheral blood, a good concordance was seen between the quantitative RT-PCR (qRT-PCR) assay results and the CellSearch[®] method (Veridex) that has been approved by the United States Food and Drug Administration (FDA) for clinical use. *KLK2* mRNAs were found in 49% of the 76 patients with metastatic castration-resistant prostate cancer, but only in 8% of the 180 men with localized cancer and never in healthy controls (Helo *et al.,* 2009).

2.3.3.2 KLK3 (kallikrein-related peptidase 3)

The protein product of the *KLK3* gene was identified in the 1970s (Hara *et al.,* 1971; Wang *et al.,* 1979) and proposed for prostate cancer diagnostics in 1980 (Papsidero *et al.,* 1980). According to the current kallikrein nomenclature, the protein is called KLK3, but the more widely-known name PSA is used in this thesis for practical reasons. Since its discovery, PSA has become the biomarker of choice for prostate cancer detection and management, despite its certain shortcomings that have been described in more detail in Chapter 2.1.5. Similar to KLK2, the main physiological function of PSA is to liquefy the seminal clot by hydrolysing the proteins in it (Lilja 1985).

The *KLK3* gene was first sequenced in 1989 (Henttu & Vihko 1989; Lundwall 1989; Riegman *et al.,* 1989) and eleven different mRNA splice variants have been identified (Kurlender *et al.,* 2005). The transcriptional level of *KLK3* expression in prostate tissue has been studied by Northern blotting and *in situ* hybridization and *KLK3* mRNA levels have been reported to be unchanged or slightly decreased in cancerous prostate tissue. Using tissue samples of 51 patients, Meng and colleagues (2002) reported an approximately 1.5 times lower *KLK3* mRNA expression in the cancerous regions than in benign epithelium. When compared to benign hyperplastic tissue, tendencies towards downregulation in cancer have been reported, but the differences have rarely

been statistically significant (Henttu *et al.,* 1990; Hakalahti *et al.,* 1993; Herrala *et al.,* 2001).

The expression of *KLK3* is largely restricted to the prostate, but low levels have been found also in salivary glands, as well as in breast and brain tissues (Lawrence *et al.,* 2010). The prostate-specific expression of *KLK3* indicated a possibility to use it as a molecular tool for prostate cancer diagnosis and staging by detecting prostate-specific circulating tumor cells in blood. After the promising initial results by Katz and Moreno and their colleagues, where transcripts were found in 33–78% of the localized or metastasized cancers but never in cancer-free controls (Moreno *et al.,* 1992; Katz *et al.,* 1994), later studies have produced contradictory data with *KLK3* mRNAs present also in the healthy category (Henke *et al.,* 1997; Thiounn *et al.,* 1997). Methodological aspects and factors related to sample cohorts and processing may have contributed to the discrepancies between the studies, and sometimes an increased sensitivity has been speculated to be the cause for the seemingly false positives in the controls. However, with a sensitive qRT-PCR assay (and similarly to *KLK2* transcripts as described in Chapter 2.3.3.2), *KLK3* mRNAs have been shown to serve as good markers for prostatic cells in the blood of prostate cancer patients. The transcripts were only detected in localized and metastatic cancer cases and the results were in concordance with findings achieved with the CellSearch® method (Veridex) (Helo *et al.,* 2009).

2.3.3.3 KLK4 (kallikrein-related peptidase 4)

KLK4 gene was originally characterized by Nelson and colleagues (1999), and at the time, its protein product was called prostase. Its biological role in humans is not fully clarified but has been suggested to be involved in tooth formation (Borgono *et al.,* 2004). At the protein level, it is claimed to be expressed in very small amounts in normal and neoplastic prostates (Obiezu *et al.,* 2002).

It has sometimes been suggested that the first kallikrein exon is not part of the functional gene as far as *KLK4* is concerned. There are reports that indicate that it would, therefore, be missing the signal peptide and have an intracellular, instead of a secretory, function (Korkmaz *et al.,* 2001; Xi 2004). *KLK4* is transcribed into at least nine different mRNA variants (Nelson *et al.,* 1999; Kurlender *et al.,* 2005). Additionally, a chimeric sense-antisense transcript has been identified (Lai *et al.,* 2010).

In addition to prostate, *KLK4* mRNA expression has been found at low to moderate levels in other tissues including adrenal, salivary, and thyroid glands, and small intestine (Nelson *et al.,* 1999; Day *et al.,* 2002). Using qRT-PCR, Day and colleagues showed initially that *KLK4* mRNA levels are equally upregulated in both normal and cancerous prostate tissues compared to the other normal human tissues. However, later studies with an *in situ* hybridization analysis of 118 tissue microarrays (Xi 2004) and a qRT-PCR analysis of 60 cancerous and 59 BPH tissues have shown upregulation in

cancerous tissues compared to normal, PIN, and BPH tissues (Avgeris *et al.,* 2011). The Avgeris study also demonstrated a positive correlation between high *KLK4* mRNA levels and cancer stage, but found no connection to Gleason score.

2.3.3.4 KLK15 (kallikrein-related peptidase 15)

KLK15 is one of the later discovered kallikreins and its biological role has not been clarified yet. In addition to prostate, *KLK15* mRNA transcripts have been found in salivary glands, testes, kidneys, colon, adrenals, and thyroid gland (Yousef *et al.,* 2001). The initial semiquantitative RT-PCR analyses revealed elevated *KLK15* mRNA levels in 45% of the 29 studied pairs of cancerous and matched noncancerous prostate tissue, and only three pairs showed lower *KLK15* mRNA levels in the cancerous counterpart. This was later repeated with qRT-PCR by Stephan and colleagues, using 90 matched cancer-noncancer tissue pairs of which 84% showed upregulation in cancer. They also demonstrated an association of higher *KLK15* expression with higher pathological stage (Stephan *et al.,* 2003).

The *KLK15* gene is alternatively spliced and at least six splice variants have been identified (Yousef *et al.,* 2001; Kurlender *et al.,* 2005). In an experiment evaluating the differential tissue expression of the classical variant and three additional transcript variants separately, Mavridis and colleagues showed that only the classical variant was overexpressed in cancer (Mavridis *et al.,* 2010). The same isoform was later specifically associated with pathological stage (Mavridis *et al.,* 2013). Higher *KLK15* levels correlated with a higher pathological tumor stage and Gleason score also in a study of 25 pairs of cancerous and matched noncancerous tissue from radical prostatectomies, regardless of the variant type as the amplicon used in the qRT-PCR assay enabled the detection of all *KLK15* mRNA variants (Rabien *et al.,* 2010).

2.3.4   MSMB (microseminoprotein, beta)

The gene *MSMB* (microseminoprotein, beta) is located in chromosome 10q11.2 (Sasaki *et al.,* 1996). It codes for the cysteine-rich beta-microseminoprotein (MSP) that has also been called PSP94 (prostate secretory protein of 94 aminoacids) or beta-inhibin (Ulvsback *et al.,* 1989). It is one of the three most abundant proteins secreted by the epithelial cells of the prostate (Lilja & Abrahamsson 1988), and it has high prostate specificity but also low expression in cervix (Baijal-Gupta *et al.,* 2000), respiratory tissues such as lung tissue (Ulvsback *et al.,* 1989), and mucosal fluids such as tears and saliva (Weiber *et al.,* 1990). Within the prostate, the expression of *MSMB* varies depending on the zone, with the highest levels in the peripheral zone where, perhaps not incidentally, most prostate tumors are found (Chan *et al.,* 1999).

At protein level, decreased MSP concentrations in serum have been linked with prostate cancer, as reviewed by Whitaker and colleagues (2010). Similar trends have been observed in urine studies (Tremblay *et al.,* 1987; Flatley *et al.,* 2014). However,

the protein and mRNA levels of *MSMB* do not always correlate, even in tissue (Tsurusaki *et al.,* 1998), and the discrepancies could be explained by differences in the half-lives of the protein and mRNAs, or the fact that the degraded forms of mRNA fail to be detected by the assays.

The mRNA expression of *MSMB* in prostate tissue has been examined by both *in situ* hybridization and qRT-PCR techniques using radical prostatectomy samples or needle biopsies. The majority of studies have found *MSMB* mRNA levels to be lowered or the mRNA to be absent in malignant tissue compared to benign samples. Vanaja and colleagues measured *MSMB* mRNA levels in 32 cancerous prostate tissues with qRT-PCR based on the Taqman method, and using *GAPDH* as a reference gene (Vanaja *et al.,* 2003). They found *MSMB* to be downregulated in malignant tissue compared to the 12 adjacent benign tissues and, furthermore, in metastatic cases compared to organ-confined cancers. Three *in situ* hybridization studies have come to the same conclusion. Chan and colleagues (1999) found a decrease in *MSMB* expression in a cohort of five tumor samples compared to 27 benign prostate tissues. The other two *in situ* hybridization studies were conducted by a group in Nagasaki, Japan, finding 63/92 and 72/104 cancerous prostate biopsies negative for *MSMB* mRNA, respectively (Tsurusaki *et al.,* 1998; Sakai *et al.,* 1999). The control tissues, five in each study, were always positive for *MSMB* mRNA expression. However, neither study found a correlation between *MSMB* expression and clinical stage or Gleason grade. Different *MSMB* mRNA variants have been identified and in a qRT-PCR study, the variant *MSMB1* was reported to be the predominant form in malignant prostate tissues whereas *MSMB2*, the variant lacking the exon 3, was primarily expressed in benign tissues (Harries *et al.,* 2010).

MSP has been shown to play a role in regulating cell apoptosis in normal cells, and therefore its loss in cancer could be postulated to lead to uncontrolled cell proliferation (Garde *et al.,* 1999). This would indicate that advanced, metastatic prostate cancers would lack *MSMB* expression, but in fact, prostate cancers with high *MSMB* expression have been linked to shorter progression-free survival (Sakai *et al.,* 1999). This is rather unexpected, but despite having been contradicted by the Vanaja study described above (Vanaja *et al.,* 2003), the finding is supported by *MSMB* expression having been found in prostate cancer cell lines LNCaP and PC3, both deriving from metastatic origins (Yang *et al.,* 1998a; Yang *et al.,* 1998b). Nevertheless, androgen-deprivation therapy decreases *MSMB* mRNA levels in prostate tissue (Dahlman *et al.,* 2010).

MSMB mRNA has been shown to have potential use as a urine-based biomarker. In a study cohort of 91 prostate cancer patients and 85 men with BPH or prostatitis, and using qRT-PCR with the LightCycler method and the normalization of the data towards a reference gene, *MSMB* was found to be able to discriminate the cancer cases from other prostatic diseases as a part of a duplex model with *TRPM8*, a triplex model with *TRPM8* and *AMACR*, or a quadriplex model with *TRPM8*, *AMACR*, and *PCA3* (Jamaspishvili *et al.,* 2011).

Serum MSP levels can be influenced by genetic variance, and a SNP located upstream of *MSMB* gene in chromosome 10q11.2 has been associated with prostate cancer (Eeles *et al.,* 2008; Thomas *et al.,* 2008). The low risk allele has a C instead of a T at the location rs10993994. It could account for the differences in MSP levels in serum as the CREB (cAMP response element-binding) transcription factor binds preferentially to the low risk allele, leading to higher MSP levels (Chang *et al.,* 2009; Waters *et al.,* 2010; Dahlman *et al.,* 2010). The risk allele T is shown to be present in 40% of Caucasians (Eeles *et al.,* 2008; Thomas *et al.,* 2008) and 60% of African-Americans (Chang *et al.,* 2011), perhaps partly accounting for the higher prevalence of prostate cancer observed in the African-American population.

2.3.5   *PCA3 (prostate cancer antigen 3)*

The majority of the human genome, 80%, is transcribed into RNA molecules without protein-coding potential. If the molecules are longer than 200 nucleotides in length, they are called long noncoding RNAs (lncRNAs). Molecules shorter than that belong to small noncoding RNAs, and microRNAs (miRNAs) that are only 20-22 nucleotides long are one of their most studied subgroups. Noncoding RNAs have been shown to function as regulators of gene expression and cellular functions. LncRNAs can often be found in the nucleus, where they regulate gene expression epigenetically by binding to chromatin remodeling complexes, and for example miRNAs can act as negative post-transcriptional regulators (Martens-Uzunova *et al*., 2014). *PCA3* (prostate cancer antigen 3) is a gene that is found to be transcribed into a lncRNA associated with prostate cancer. *PCA3* was discovered in 1999 and was first named DD3 (differential display 3) according to the discovery technique (Bussemakers *et al.,* 1999). It has been mapped to chromosome 9q21.22, and its RNA product is currently, despite research efforts, not known to code for any functional protein, as it has very short open-reading frames. *PCA3* transcripts have been localized to the nucleus (Schalken *et al.,* 2003) and a study with small interfering RNAs (siRNAs) has indicated a potential role for *PCA3* in the regulation of androgen receptor target genes (Ferreira *et al.,* 2012). Initially, the *PCA3* gene was described to comprise of four exons (Bussemakers *et al.,* 1999), with two additional ones discovered ten years later (Clarke *et al.,* 2009). Alternative splicing of the gene occurs, with the predominant transcript consisting of exons 1, 3, 4a and 4b (Bussemakers *et al.,* 1999).

Already in the initial studies, the expression of *PCA3* was demonstrated to be predominantly prostate-specific as no expression was detected even by RT-PCR in any of the other cancer types studied or in the 18 normal human tissue types, with the exception of low levels in kidney tissue (Bussemakers *et al.,* 1999; de Kok *et al.,* 2002). The absence of *PCA3* transcripts in leukocytes suggested potential usefulness in detecting prostate cells in bodily fluids such as blood and urine (de Kok *et al.,* 2002).

Using Northern blotting, the upregulation of *PCA3* was associated with prostate cancer with 95% of the 56 tumors overexpressing *PCA3* in comparison to matched

nonneoplastic tissue, even though low levels of *PCA3* RNA were seen in normal prostate and BPH tissue with RT-PCR. Since then, variable magnitudes of *PCA3* RNA overexpression have been reported in cancerous prostate tissue, ranging from 10 to 100-fold upregulation (Bussemakers *et al.,* 1999; de Kok *et al.,* 2002; Hessels *et al.,* 2003; Robert *et al.,* 2013), or 6–1500-fold overexpression in individual matched cases (Hessels *et al.,* 2003). The observations are summarized in Table 3. In a review by Schalken and colleagues, the *PCA3* RNA copy levels are reported to be 3.2×10^2–8.5×10^5 copies/µg RNA in normal prostate or BPH tissue, and 5.3×10^4–1.4×10^8 copies/µg RNA in cancerous tissue (Schalken *et al.,* 2003).

Table 3. Magnitudes of *PCA3* overexpression observed in cancerous prostate tissue.

Reference	Carcinoma samples (n)	Control samples (n)	Method of analysis	Magnitude of *PCA3* overexpression
Bussemakers *et al.,* 1999	56	Benign tissues from the same PCa patients	Northern blotting	10–100-fold
de Kok *et al.,* 2002	31	11 benign tissues from PCa patients + 5 BPH from PCa patients	qRT-PCR	34-fold
Hessels *et al.,* 2003	27 (containing more than 10% cancer cells)	4 benign tissues from RP specimens + 8 BPH	qRT-PCR	66-fold
	13 (containing less than 10% cancer cells)	4 benign tissues from RP specimens + 8 BPH	qRT-PCR	11-fold
	7	Benign tissues from the same PCa patients	qRT-PCR	6–1500-fold
Robert *et al.,* 2013	48	32 benign tissues from PCa patients	qRT-PCR	30-fold

PCa, prostate cancer; BPH, benign prostatic hyperplasia; qRT-PCR, quantitative reverse-transcription PCR; *PCA3*, prostate cancer antigen 3.

The promising initial and subsequent data on the prostate cancer specificity of *PCA3* generated a lot of interest in the field, and subsequently led to a commercial application of a noninvasive urine-based test for *PCA3* RNA levels, currently offered under the brand name of PROGENSA® PCA3 (Gen-Probe) (Groskopf *et al.,* 2006). It measures the *PCA3* RNA content of cells in a post-DRE urine sample. The DRE procedure has been shown to increase the dissociation of the prostate cells into urine, and the informative rate (the percentage of samples having enough prostate cells to warrant the molecular analysis) increases from 80% to 95% with DRE (Day *et al.,* 2011). The presence of prostate cells is controlled in the *PCA3* test by measuring the *KLK3* mRNA transcript level from each sample simultaneously with the *PCA3* RNA level. The

PROGENSA® PCA3 test was launched as a semiquantitative, commercially available test in Europe in 2006 and received approval from the United States FDA in 2012. It is performed on whole urine, and the analysis is based on capturing the target RNAs with magnetic beads, followed by transcription-mediated amplification of the RNAs, and the detection of the amplification by chemiluminescent probes. The urine centrifugation and RNA extraction steps can be bypassed using this protocol, and the voided urine is stored in an equal volume of detergent-based stabilization buffer, enabling the freezing of the sample. The test detects an amplicon that spans the junction of exons 3 and 4a, thereby recognizing all of the known *PCA3* transcripts (Groskopf *et al.,* 2006).

In cohorts of 108 and 517 men, the first and second-generation versions of the PROGENSA® PCA3 test, respectively, produced sensitivity and specificity values of 66–82% and 76–89% (Hessels *et al.,* 2003; Fradet *et al.,* 2004; Tinzl *et al.,* 2004). The concept of the PCA3 score, the ratio of *PCA3*/*KLK3* transcripts multiplied by a factor of 1000, obtained from the 3rd generation *PCA3* test was first introduced by Groskopf and colleagues (Groskopf *et al.,* 2006) and reported under that name by van Gils and colleagues (van Gils *et al.,* 2007). These studies demonstrated sensitivities of 65–69% and specificities of 66–79%. Since then, the performance of the PROGENSA® PCA3 test has been validated in diverse laboratories, and higher median PCA3 scores in men with prostate cancer have been repeatedly demonstrated (Marks *et al.,* 2007; Haese *et al.,* 2008; Deras *et al.,* 2008; Aubin *et al.,* 2010). Notably, the test also produces consistently better area under the curve (AUC) values in receiver operating characteristic (ROC) analyses than those obtained with serum PSA levels for the same cohorts (Marks *et al.,* 2007; Haese *et al.,* 2008), but the scores are associated with prostate volume unlike serum PSA (Haese *et al.,* 2008; Deras *et al.,* 2008). As reviewed by Day and colleagues, the PCA3 score contributes to improved diagnostic accuracy in multivariate models (Day *et al.,* 2011). The correlation between PCA3 score and high-grade PIN found at biopsy is still under investigation due to contradictory data (Deras *et al.,* 2008; Haese *et al.,* 2008). The prognostic value of PCA3 score is also still unclear, as studies have reported varying results on the association with Gleason score and tumor volume (Haese *et al.,* 2008; van Gils *et al.,* 2008; Nakanishi *et al.,* 2008).

Initially a PCA3 score of 35 was recommended as the cut-off point for an increased risk of having cancer at repeat biopsy (Marks *et al.,* 2007; Haese *et al.,* 2008). Using this cut-off of 35, sensitivities of 48–58% and specificities of 72–79% have been reported (Marks *et al.,* 2007; Haese *et al.,* 2008; Deras *et al.,* 2008; Aubin *et al.,* 2010). The FDA-approved test uses a cutoff of 25, but also other cut-off values have been suggested to avoid false negative results (de la Taille *et al.,* 2011; Crawford *et al.,* 2012). However, there are also recent studies by Schröder and colleagues demonstrating that cohorts of men with PCA3 scores ≥100 can show surprisingly low rates of detected cancers, even after several rounds of biopsies and a magnetic resonance imaging (MRI) scan (Roobol *et al.,* 2010; Schröder *et al.,* 2014). Rates as

low as 11% were seen in a cohort of 62 men. Earlier, the risk of having a positive biopsy was reported to be 46–89% in men with PCA3 scores over 100 (Marks *et al.,* 2007; van Gils *et al.,* 2007; Deras *et al.,* 2008; Haese *et al.,* 2008; Shappell *et al.,* 2009). It has been speculated that abnormally high PCA3 scores in seemingly cancer-free men may be caused by the upregulation of *PCA3* in precursor lesions, or simply cancers missed by biopsy (Schröder *et al.,* 2014). In fact, *PCA3* expression has been detected by *in situ* hybridization more frequently in benign glands that are in closer proximity to the tumor than in glands further away, demonstrating that alterations in *PCA3* expression can appear in benign regions in association with or anticipation of cancer (Popa *et al.,* 2007). This could be explained by the field cancerization theory, i.e., that molecular aberrations are not limited to the tumor focus but may be present in a larger region (Slaughter *et al.,* 1953).

A small number of studies have attempted the detection of *PCA3* transcripts in circulating tumor cells in peripheral blood. Immunomagnetic enrichment was used to increase sensitivity in studies by Shaw and colleagues, and Jost and colleagues (Shaw *et al.,* 2008; Jost *et al.,* 2010). Both found *PCA3* RNA in blood of men with advanced or castration-resistant prostate cancer. All studied men presented *PCA3* transcripts in the Shaw study, and also one of the healthy controls did so as well, albeit at a low level. In the Jost study, no *PCA3* transcripts were found in cases of androgen-dependent cancers. Studies performed without a cell enrichment step have reported *PCA3* RNA findings in blood at least twice. Nested RT-PCR combined with a densitometric analysis of the amplification products from an agarose gel detected *PCA3* RNA in 14/84 cancer cases, 13/65 men with BPH, and one out of 11 studied men with PIN diagnosis (Marangoni *et al.,* 2006). In a later study using a similar method, *PCA3* transcripts were found in the blood of 28/57 men with T1–T3 stage prostate cancer and 6/110 healthy individuals (Neves *et al.,* 2013). Surprisingly, the positivity rate, especially among cancer-free men, was higher in the studies where cell enrichment was not used. This may be related to other methodological aspects or, as speculated by the authors, a sign of a cancer risk in the future.

2.3.6   *PSCA (prostate stem cell antigen)*

PSCA (prostate stem cell antigen) gene is located in chromosome 8q24.2 and it codes for a cell surface glycoprotein that was identified as a candidate biomarker for prostate cancer in 1998 (Reiter *et al.,* 1998). The mRNA expression of *PSCA* was deemed to be prostate-specific after analyses with Northern blotting and RT-PCR showed that it is predominantly found in prostate with only low level expression in placenta, kidneys, and small intestine. The physiological function of PSCA protein remains unknown (Saeki *et al.,* 2010; Bargão & Patel 2014).

In prostate, *PSCA* mRNA expression was shown by *in situ* hybridization to be localized to the basal cell layer of the epithelium, and 88% of the 126 cancerous prostate tissue specimens and 82% of the 118 high-grade PIN samples stained strongly

or moderately for *PSCA* mRNA, while normal glands of the same specimens stained only weakly or not at all (Reiter *et al.,* 1998). However, later studies, also performed with *in situ* hybridization techniques, have contradicted the results of the pilot study and showed that *PSCA* mRNA can also be found in the secretory luminal cells (Ross *et al.,* 2001; Ross *et al.,* 2002; Zhigang & Wenlv 2004) and, in fact, also in normal areas of the prostate, without apparent association between the expression level and the tissue morphology (Ross *et al.,* 2001; Ross *et al.,* 2002). The later reports also disagree on the association of the neoplasticity of the cells with the levels or frequencies of *PSCA* mRNA expression. A study by Ross and colleagues found no apparent association between the expression level and tissue morphology (Ross *et al.,* 2002), but another study reported higher expression levels in prostate cancer and high-grade PIN tissue compared to those in BPH or low-grade PIN tissue (Zhigang & Wenlv 2004). Both studies were performed with *in situ* hybridization. A study utilizing the more sensitive qRT-PCR method did not find statistically significant differences between *PSCA* mRNA levels in cancerous and matched nonmalignant prostate tissue (Schmidt *et al.,* 2006b).

PSCA mRNA has not been detected in the peripheral blood of healthy individuals, but the prevalence in men with prostate cancer varies from 10 to 32% (Hara *et al.,* 2002; Joung *et al.,* 2007; Helo *et al.,* 2009; Zhao *et al.,* 2012). Hara and colleagues determined the expression of *PSCA* mRNA in the circulating tumor cells in prostate cancer patients by nested RT-PCR combined with detection from agarose gel, and found it in the blood of 13.8% of the 58 studied prostate cancer patients but in none of the blood specimens from 71 men with nonmalignant diseases (Hara *et al.,* 2002). In 2007, *PSCA* mRNA was detected also with RT-PCR in the peripheral blood of 13.6% of 66 prostate cancer patients. A significantly higher number of those patients had high-grade cancer or non-organ-confined cancer than low-grade or organ-confined disease, and it was concluded that *PSCA* mRNA in blood could help in the molecular staging of prostate cancer (Joung *et al.,* 2007). The qRT-PCR assay developed in this doctoral work has also been used elsewhere for determining *PSCA* mRNA-positive cells in the blood of prostate cancer patients, and 10% of castration-resistant stage patients were found to be positive for *PSCA* mRNA (Helo *et al.,* 2009), whereas the transcripts were undetectable in cases of localized cancer and in healthy volunteers. Zhao and colleagues recently detected *PSCA* mRNA in the blood of 31.9% of 116 patients with locally advanced or metastatic disease but in none of the 40 healthy volunteers (Zhao *et al.,* 2012).

Zhigang and colleagues have studied the role of *PSCA* mRNA as a predictor of cancer development. With *in situ* hybridization, they noticed that *PSCA* mRNA expression in the PIN or BPH tissue of a cancer-free prostate predicted a later development of prostate cancer (Zhigang & Wenlu 2007; Zhigang & Wenlu 2008). A cohort of 288 BPH cases with serum PSA levels under 4.0 ng/mL were followed for up to 70 months, and 23.7% of the 93 originally *PSCA*-positive patients developed cancer during the follow-up time, as opposed to only 2 of the 195 *PSCA*-negative cases (Zhigang &

Wenlu 2008). A cohort of 166 BPH patients that were considered to have an increased cancer risk and had serum PSA levels higher than 4.0 ng/mL demonstrated even more remarkable results: 73.8% of the 42 patients who had been *PSCA* mRNA-positive at the preoperative biopsy at the beginning of the study, developed prostate cancer during the study period of five years, whereas none of the 124 PSCA-negative patients developed cancer (Zhao *et al.,* 2009). In a cohort of 117 men with PIN at initial biopsy, there was a significantly higher frequency of *PSCA* mRNA expression observed in the high-grade PIN specimens compared to the low-grade PIN samples, and the expression was significantly elevated in those men that subsequently developed cancer (Zhigang & Wenlu 2007). Thirty-four cases overall developed cancer during a follow-up time of up to 85 months and all cancer cases expressed *PSCA*. The subsequent cancer was significantly more likely to develop if the original specimen was *PSCA* mRNA positive.

There are indications that the blood level of *PSCA* mRNA may perform as a predictor of biochemical relapse. Joung and colleagues were able to detect *PSCA* mRNA in the blood of 17 of the 103 studied prostate cancer patients before their curative radical prostatectomies, and they monitored the patients for up to 47 months for biochemical relapse (Joung *et al.,* 2010). Of the 17 *PSCA*-positive cases, 41.2% relapsed as opposed to 23.3% of the *PSCA*-negative patients.

A recent study showed that the silencing of *PSCA* gene with siRNAs arrests the growth of cells of a human prostate cancer cell line PC-3M and restricts their invasiveness and proliferation (Zhao *et al.,* 2013). This implies a possibility to utilize *PSCA* as a therapeutic modality.

2.3.7   SPINK1 (serine peptidase inhibitor, Kazal type 1)

Paju and colleagues first showed that tumor-associated trypsin inhibitor (TATI), the protein product of serine peptidase inhibitor Kazal type 1 (*SPINK1*) gene located in chromosome 5 (Horii *et al.*, 1987), is expressed in prostate tissue in both benign and malignant regions, and the results were confirmed at mRNA level by RT-PCR (Paju *et al.,* 2007). All BPH tissues also stained at least weakly for TATI in immunohistochemistry experiments. Studies have indicated a growth factor role for SPINK1, and based on murine experiments, it is also suggested to act as a trypsin inhibitor and autophagy regulator (Ohmuraya & Yamamura 2011).

Since the initial prostate-related study, several studies have linked the upregulation of TATI with prostate cancer, but reports on *SPINK1* at the mRNA transcript level are infrequent and mostly limited to urine-based studies. Sabaliauskaite and colleagues (2012) examined *SPINK1* transcript levels in 158 prostate carcinoma tissues and 21 noncancerous prostatic tissue samples by qRT-PCR, but did not see any significant up- or downregulation in cancer patients.

Reports on the usefulness of *SPINK1* transcript detection in urine are contradictory. In a study by Laxman and colleagues (2008), urine of men with suspected prostate cancer was measured for *SPINK1* mRNA expression among other markers, and *SPINK1* was shown to be useful for discriminating cancer patients from biopsy-negative men both on its own and as a part of a multiplex setting with *PCA3*, *TMPRSS2-ERG* and *GOLPH2* transcripts. However, in a recent study by Jamaspishvili and colleagues (2011), these results were not reproduced, and a model of four best-performing markers (*AMACR*, *PCA3*, *TRPM8* and *MSMB*) functioned equally well in comparison to a full model that included *SPINK1*. A study by Dimitriadis and colleagues (2013) evaluating *SPINK1* transcript levels in the urine of 66 men scheduled for prostate biopsies also failed to show any benefits of urinary *SPINK1* detection. Fourteen cases of cancer were subsequently found, but the ratio of *SPINK1/KLK3* mRNA levels was not significantly different between the cases and controls (Dimitriadis *et al.,* 2013).

A specific aspect of *SPINK1* expression that has generated interest recently, is its claimed mutual exclusivity with the *TMPRSS2-ERG* genomic rearrangement. TATI expression, detected by immunohistochemistry, has been found exclusively (Tomlins *et al.,* 2008; Bismar *et al.,* 2012) or at least more frequently (Han *et al.,* 2009; Bhalla *et al.,* 2013; Grupp *et al.,* 2013; Leinonen *et al.,* 2013) in cancer cases without the *TMPRSS2-ERG* fusion gene. Studies have spanned cohorts consisting of both castration-resistant and localized prostate cancers. The mutual exclusivity was shown recently even at the level of individual cells by Lippolis and colleagues (2013). However, in a study on 186 hormonally-treated prostate cancers, this association was absent (Leinonen *et al.,* 2010). On a rare study performed at the mRNA transcript level, tissue from 28 cancerous prostates demonstrated outlier pattern in *SPINK1* mRNA expression in cancer types that lacked the ERG rearrangement (Jhavar *et al.,* 2009). This was a genome-wide expression study performed on microarrays. All in all, the possibility of a subgroup of *TMPRSS2-ERG* negative prostate cancers presenting high *SPINK1* expression creates an interesting target for research.

2.3.8   *TMPRSS2-ERG fusion gene*

The fusion between *TMPRSS2* (transmembrane protease serine 2) and *ERG* (ETV-related gene) genes was identified in 2005 to be a recurring event in prostate cancer (Tomlins *et al.,* 2005). Other fusion partners for the androgen-regulated, prostate-specific *TMPRSS2,* such as *ETV1*, *ETV4,* and *FLI1,* have later been discovered, but *ERG* remains the most prevalent of them (Tomlins *et al.,* 2005; Tomlins *et al.,* 2009; Paulo *et al.,* 2012).

The genes are located on the same chromosome, *TMPRSS2* in locus 21q22.2 and *ERG* in 21q22.3. Over 20 different fusion variants have been identified (Tomlins *et al.,* 2009), and individual tumors have been shown to be able to contain more than one of these variants (Furusato *et al.,* 2008). There are different potential fusion breakpoints, and alternative splicing creates additional variants (Clark *et al.,* 2007), but the basic

consensus is that the fusion joins together the promoter region of *TMPRSS2* and the coding region of the *ERG* gene. The most common isoform, called *TMPRSS2-ERG* III or *TMPRSS2-ERG* a (Wang *et al.,* 2006; Furusato *et al.,* 2008), consists of the exon 1 of *TMPRSS2* and the exons of ERG coding region from exon 4 onward. Due to the fact that *TMPRSS2* is regulated by androgens, the gene fusion can lead to a prostate-specific overexpression of the transcription factor ERG, possibly in a truncated form. Approximately 90% of the prostate cancers with ERG overexpression have presented the *TMPRSS2-ERG* fusion (Tomlins *et al.,* 2005). In addition to the fluorescence *in situ* hybridization (FISH) break-apart assay originally used to detect the gene rearrangement, and RT-PCR assays measuring the fusion mRNA transcripts, the detection of the ERG protein by immunohistochemistry is one of the possible gene fusion identification methods (Tomlins *et al.,* 2005; Park *et al.,* 2010). Studies have compared the abilities of the FISH and RT-PCR assays to identify the presence of the fusion gene, and concordances higher than 80% between the methods have been reported (Tu *et al.,* 2007; Fernandez-Serra *et al.,* 2013). These earlier experiments were performed on FFPE tissues in which, conceivably, the RNA quality may have been compromised, but Svensson and colleagues, using fresh frozen biopsy tissue, recently found a good concordance between the *TMPRSS2-ERG* FISH assay and a transcription-mediated assay for the fusion gene mRNA (Svensson *et al.,* 2014).

The *TMPRSS2-ERG* gene fusion has been claimed to be a cancer-specific rearrangement, and present in a subset of prostate cancers. In the initial studies using FISH and RT-PCR, this rearrangement was found in the prostate tissue of 30–50% of localized prostate cancers and in 19–21% of PIN tissues, but in none of the control tissues that had been obtained from BPH, prostates with inflammation, or histologically benign tissue of cancerous or cancer-free prostates (Cerveira *et al.,* 2006; Perner *et al.,* 2007; Attard *et al.,* 2008). The frequency of the fusion gene was found to be similar among localized and metastasized cases of prostate cancer. By FISH analyses, the rearrangement was seen in 42–49% of localized prostate cancers and in 30–41% of metastatic cases (Perner *et al.,* 2006; Perner *et al.,* 2007; Gopalan *et al.,* 2009). Later, the subgroup showing the fusion has been suggested to represent roughly half, or 40–70% of all prostate cancers (Tomlins *et al.,* 2009). A higher frequency has been observed in Caucasians than in African-American, Indian or Japanese cohorts (Magi-Galluzzi *et al.,* 2011; Rawal *et al.,* 2013).

The initial findings generated a lot of enthusiasm in the research related to *TMPRSS2-ERG*. Despite the fact that its functions are still not fully understood, knowledge on the causative factors and consequences has increased rather rapidly, and ten years later, the annual number of new publications on the subject is still rising exponentially (Figure 2). This is conceivable as many aspects of the biology and potential relevance of the fusion are still unclear, and the correlation between the fusion gene or its specific variants and prostate cancer prognosis are still debated.
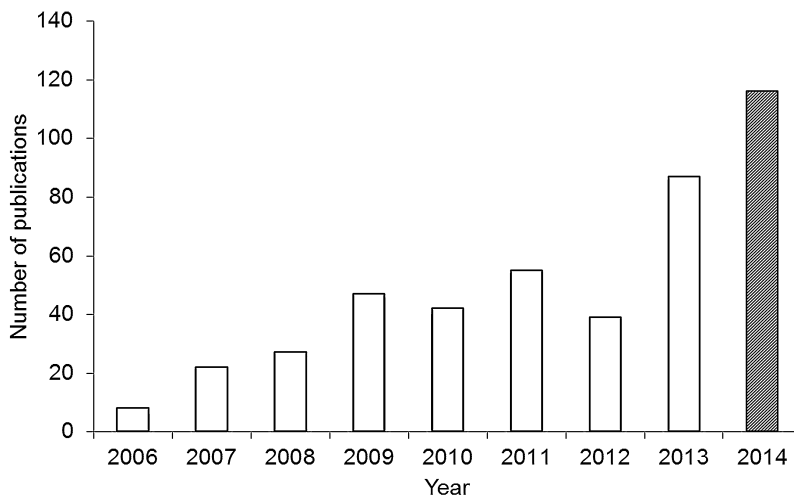
Figure 2. The number of new *TMPRSS2-ERG* related publications in PubMed database after the publication of the initial study. The number for the current year, 2014, was estimated based on the number of reports published during the first quarter of the year.

The association between the fusion gene in general and poorer survival has sometimes been reported (Attard *et al.,* 2008), but that observation has not been evident in all studied cohorts (Gopalan *et al.,* 2009). An isoform associated with aggressive cancer (*TMPRSS2-ERG* VI) was identified early, but the data have not been conclusively repeated (Wang *et al.,* 2006). Another specific variant called *TMPRSS2*(exon(0))-*ERG* has been associated with a good cancer prognosis (Hermans *et al.,* 2009).

It has been suggested that the actual fusion process might be important to the prognosis. All studied castration-resistant, metastatic cancers presented fusion through deletion instead of translocation, but only 39–60% of the localized cancers were of the deletion subtype (Mehra *et al.,* 2008). The aggressive forms have been found to be associated with fusion through deletion also in other studies (Han *et al.,* 2009; Perner *et al.,* 2006); especially the subtype where the area of fusion through deletion has been duplicated is associated with poor survival (Attard *et al.,* 2008; Gopalan *et al.,* 2009).

The mechanism that introduces the fusion has been examined and androgen has been shown to be able to induce the proximity of *TMPRSS2* and *ERG*, thereby facilitating the fusion in case of a subsequent double-strand break in DNA. This has been shown in both nonmalignant cells and in LNCaP, the prostate cancer cell line derived from a lymph nodal metastasis, that does not inherently harbor the fusion gene (Mani *et al.,* 2009; Bastus *et al.,* 2010; Haffner *et al.,* 2010).

In addition to tissue-level studies, *TMPRSS2-ERG* transcript detection has also been applied to noninvasive settings. Among the first researchers, Laxman and colleagues (2006) detected the fusion mRNA by qRT-PCR in the post-prostatic massage urine of 42% of the 19 prostate cancer patients. Hessels and colleagues (2007) used semiquantitative RT-PCR combined with Southern blotting to find the fusion transcripts in a cohort of 108 men. Of the 78 men who were diagnosed with prostate cancer at biopsy, 37% were found to have *TMPRSS2-ERG* mRNA in their urine. This mirrors the rate found in the Laxman study as well as the frequency observed in prostate tissues.

The initially claimed high cancer specificity of *TMPRSS2-ERG* has later been questioned in a few studies that have demonstrated *TMPRSS2-ERG* transcript expression in BPH tissue or histologically benign tissue in cancerous prostates. Clark and colleagues (2007) first reported finding the fusion mRNA in 8/17 cancer-adjacent benign tissues. These data were later repeated by Furusato, Sabaliauskaite, and Robert with their colleagues, with 2/45, 4/21, and 5/32 cancer-adjacent benign tissues, respectively, showing *TMPRSS2-ERG* expression (Furusato *et al.,* 2008; Sabaliauskaite *et al.,* 2012; Robert *et al.,* 2013). The Robert study also found the fusion transcripts in 8% of the 48 studied BPH tissues. Occasionally, *TMPRSS2-ERG* has also been detected in urine in the absence of cancer cells at subsequent needle biopsies (Hessels *et al.,* 2007; Nguyen *et al.,* 2011; Bories *et al.,* 2013; Leyten *et al.,* 2014). These results, combined with the findings of *TMPRSS2-ERG* in PIN, suggest that the gene fusion is an early event in the neoplastic process. The rate of false-negative biopsies could also indicate that cancer could be present and has just been missed by the biopsy procedures. In fact, in the Ngyuen study, one of the *TMPRSS2-ERG* positive men with *TMPRSS2-ERG* positive urine was diagnosed with prostate cancer at a repeat biopsy. Additionally, in a study where 461 men with high-grade PIN were evaluated for ERG expression by immunohistochemistry, it was shown that a higher proportion of ERG-positive men developed cancer within a follow-up time of three years (Park *et al.,* 2014).

2.3.9   TRPM8 (transient receptor potential melastatin 8)

Ca^{2+} signaling is known to play a role in the apoptosis, differentiation and proliferation of cells, and in cellular functions such as secretion. The entry of calcium is controlled by ion channels in the plasma membranes (Flourakis & Prevarskaya 2009). *TRPM8* (transient receptor potential melastatin 8) was identified as a predominantly prostate-specific gene coding for a protein that has transmembrane domains, and acts as a Ca^{2+} permeable ion channel. The gene is located in chromosome 2q37 (Tsavaler *et al.,* 2001; Zhang & Barritt 2004). Of other normal tissues, only testis tissue shows a low level of *TRPM8* mRNA expression with a trace-amount expression seen in lung, breast, thymus, colon, liver, and small intestine tissues (Tsavaler *et al.,* 2001; Cunha *et al.,* 2006).

TRPM8 expression is related to androgens (Zhang & Barritt 2004; Bidaux *et al.,* 2005; Bai *et al.,* 2010), and the differentiation status of the cells. In differentiated cells of the prostate epithelium, the TRPM8 channels can be found in plasma membranes or in the endoplasmic reticulum, but the plasma membrane-specific expression is lost in poorly differentiated cells (Bidaux *et al.,* 2007). However, the prostate-specific function of the TRPM8 channel in normal or cancerous conditions is not fully clear (Flourakis & Prevarskaya 2009). Somehow the inhibition of the channel expression or the channel function slows down the growth of malignant prostate cells, but does not have an effect on normal prostate cells (Valero *et al.,* 2012).

With both *in situ* hybridization and RT-PCR methods, *TRPM8* mRNA has been found to be expressed in normal prostate tissue but also, at a higher level, in cancerous prostate tissue and in cancerous tissues from other organs (Tsavaler *et al.,* 2001; Fuessel *et al.,* 2003; Bai *et al.,* 2010). Using qRT-PCR, it was shown that *TRPM8* transcripts are significantly overexpressed in cancerous lesions compared to matched nonmalignant prostate tissue with a 3.7 times higher expression in cancer tissue (Schmidt *et al.,* 2006b). However, a subsequent decrease or loss of *TRPM8* mRNA expression in tissue in more advanced stages of cancer has been demonstrated (Henshall *et al.,* 2003; Schmidt *et al.,* 2006b). This seems to suggest that despite an increase at the onset of cancer, *TRPM8* expression has a protective role, hindering the cancer progression to advanced stage. This is supported by the finding that the activation of the TRPM8 channels by PSA reduces the motility of PC3 cells (Gkika *et al.,* 2010). Hence, the loss of TRPM8 can lead to increased cell migration.

Measurements of *TRPM8* mRNA transcripts in urine have demonstrated higher transcript levels in metastatic patients than in patients with localized disease, whereas they have failed to discover a significant difference between healthy men and men with localized cancer (Bai *et al.,* 2010). In the same study, a similar trend was seen in peripheral blood specimens; this is more likely to be caused by a higher prostate cell count in these bodily fluids than upregulated *TRPM8* expression. In a separate study on multiple biomarkers, and similarly to *MSMB* as described in Chapter 2.3.4, the *TRPM8* transcript level was reported to enhance a multiplex model for the detection of early prostate cancer in urine samples (Jamaspishvili *et al.,* 2011).

2.3.10 Summary on candidate genes

As discussed in Chapter 2.3, previous works by other researchers have indicated that connections exist between prostate cancer and the expression of the 12 candidate biomarker genes that were studied in this doctoral work. They are summarized in Table 4.

Table 4. Connections found between the 12 candidate genes and prostate cancer.

Candidate gene	Suggested connection to prostate cancer
BMP-6	Upregulated in cancer, particularly in metastasized cases.
FGF-8b	Upregulated in cancer.
KLK2	Highly expressed in prostate; Upregulated in cancer.
KLK3	Highly expressed in prostate; Constantly expressed or slightly downregulated in cancer; Produces PSA that often leaks to circulation in prostatic diseases.
KLK4	Upregulated in cancer; Correlation with cancer stage.
KLK15	Upregulated in cancer; Correlation with cancer stage.
MSMB	Highly expressed in prostate; Downregulated in cancer.
PCA3	Highly upregulated in cancer.
PSCA	Upregulated in cancer and high-grade PIN.
SPINK1	Upregulated in cancer; Overexpressed predominantly in cancers where *TMPRSS2-ERG* fusion is absent.
TMPRSS2-ERG	Rearrangement found only in men with prostate cancer and more rarely, with BPH.
TRPM8	Upregulated at the onset of cancer, decrease in expression in advanced stages.

PSA, prostate specific antigen; PIN, prostatic intraepithelial neoplasia; BPH, benign prostatic hyperplasia.

3 AIMS OF THE STUDY

The main objective of this doctoral work was to evaluate the suitability of 12 candidate genes for prostate cancer diagnostics by measuring their expression levels in prostate tissue and peripheral blood of men with and without prostate cancer. Novel, highly sensitive, and internally standardized qRT-PCR assays were developed for the accurate detection of the mRNAs produced by the target genes.

More specifically, the aims were:

I   To evaluate the alterations in *PCA3* and *KLK3* gene expression in the blood of men with localized prostate cancer, metastasized prostate cancer, other prostatic diseases, and healthy individuals by measuring their mRNA levels in circulating cells with novel qRT-PCR assays.

II   To assess the differences in the mRNA expression of ten biomarker candidates of prostate cancer – *BMP-6*, *FGF-8b*, *KLK2*, *KLK3*, *KLK4*, *KLK15*, *MSMB*, *PCA3*, *PSCA* and *TRPM8* – between cancerous and histologically benign tissue of prostate cancer patients with novel qRT-PCR assays.

III   To study the differences in the mRNA expression levels of *TMPRSS2-ERG* fusion gene, *SPINK1,* and *PCA3* in prostate tissue of men with and without clinical prostate cancer with novel qRT-PCR assays.

IV   To examine, with reference to the location of the carcinoma foci in the prostate, the alterations in the expression of *PCA3* and *TMPRSS2-ERG* mRNAs as well as that of ERG protein in the prostates of men with prostate cancer using qRT-PCR assays and immunohistochemistry.

4 SUMMARY OF MATERIALS AND METHODS

The detailed descriptions of the materials and methods used in this study are presented in the original publications (**I–IV**). A brief summary along with a few additional details is provided here.

4.1   Clinical samples

Clinical samples were obtained from participants with their informed consent. The cohorts are summarized in Table 5 and described in more detail in Chapters 4.1.1–4.1.4. The study protocols were approved by the local ethics committees and they were in accordance with the Helsinki Declaration of 1975, as revised in 1996.

Table 5. Summary of the clinical samples used in this doctoral work.

Collection site	Study population	Sample matrix	Number of samples	Publication
TUH, Turku, Finland	Men with prostate cancer	Blood	67	I
TUH, Turku, Finland	Men with nonmalignant prostatic diseases	Blood	8	I
DBUT, Turku, Finland	Healthy male and female volunteers	Blood	16	I
TUH, Turku, Finland	Men with localized prostate cancer	Tissue from RP specimens	174	II, III
SUH, Malmö, Sweden	Men with urinary bladder cancer	Tissue from CP specimens	19	III
TUH, Turku, Finland	Men with localized prostate cancer	Tissue from RP specimens	5	IV

TUH, Turku University Hospital; DBUT, Department of Biotechnology, University of Turku; SUH, Skåne University Hospital; RP, radical prostatectomy; CP, cystoprostatectomy.

4.1.1 Peripheral blood from prostate cancer patients and healthy individuals (I)

Peripheral blood was obtained from 67 men with prostate cancer, one patient with benign prostatic hyperplasia (BPH), and seven patients with prostatic infection from Turku University Hospital, Turku, Finland. Blood was also drawn from 16 healthy volunteers. From each individual, 2.5 mL of blood were collected into PAXgene™ Blood RNA tubes (PreAnalytix) containing the RNA stabilizing solution, mixed by inverting the tube and stored at –20 °C. Either before or after storage, the sample was

incubated for 24 h at room temperature according to the manufacturer's instructions before further processing.

### 4.1.2   Prostate tissue from prostate cancer patients (I, II, III)

Prostate tissue was collected from 87 men who had undergone radical prostatectomy due to clinically localized prostate cancer at Turku University Hospital, Turku, Finland. Two small samples were obtained from each fresh prostatectomy specimen immediately after surgery, one aimed to sample the cancerous area and the other a control site. The tissue area adjacent to samples was later examined microscopically by an experienced pathologist for histology and samples were subsequently classified as cancerous, PIN, or histologically benign tissue. Of the 174 samples, 88 were classified as histologically benign tissue, 86 as cancerous samples, and 12 samples fell into the PIN category but were considered as noncancerous samples in further analyses unless stated otherwise. Sample tissues were stored in a solution 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 they were stored at –80 °C until further processing.

4.1.3   Prostate tissue from bladder cancer patients (III)

Prostate tissue was collected from 19 men who had undergone cystoprostatectomy due to urinary bladder cancer at Skåne University Hospital, Malmö, Sweden. One sample was obtained from each cystoprostatectomy specimen and the prostate was further sectioned and examined pathologically to inspect the gland for incidental cancer foci. Seven glands were classified as tumor-free whereas 12 were deemed to contain incidental tumor foci in prostate regions other than the sampled area. Samples were stored fresh frozen at –80 °C until further processing.

4.1.4   Prostate tissue from cross-sections of prostate cancer patients (IV)

Prostate tissue from single cross-sections of the prostate was collected from five men, who had undergone radical prostatectomy due to prostate cancer at the Turku University Hospital, Turku, Finland. A slice of approximately 2 mm in thickness was cut horizontally from each prostate, and further cut into 5x5x2 mm pieces using a Styrofoam plate grid as a template (Figure 3). Hence, 44–62 pieces were obtained from each prostatectomy cross-section, depending on the size of the gland. Samples were stored in RNA stabilizing solution, RNAlater® (Ambion), at –20 °C until further processing. Tissue sections next to the slice from both the superior and inferior side of the prostate were examined histologically by a pathologist to determine carcinoma, PIN, and histologically benign areas and to match the tissue histology with the corresponding samples of the mRNA experiments performed later. Tissue next to the sections undergoing the histological examination was fixed in formalin and embedded in paraffin to create macro blocks of tissue for immunohistochemistry experiments.
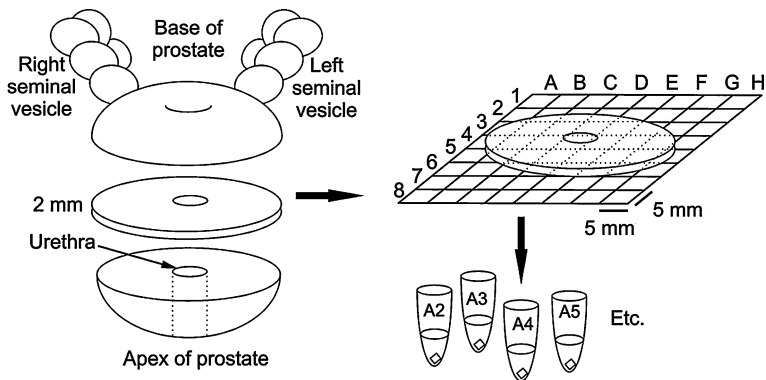
Figure 3. The sample collection flowchart of the cross-section study. A horizontal cross-section was cut from each studied prostate and further divided into 2x5x5 mm pieces that were used for mRNA measurements. (Figure from original manuscript **IV**.)

4.2   RNA extraction and reverse transcription (I, II, III, IV)

Total RNA was extracted from the blood samples using PAXgene Blood RNA kit (Preanalytix) according to the manufacturer's instructions, and a known, fixed amount of artificial RNA (Nurmi *et al.,* 2000b) was added to each sample after cell lysis to act as an internal standard. During the extraction, samples were treated with RNAse-free DNAse I (Qiagen) according to manufacturer's instructions to degrade and eliminate the contaminating genomic DNA.

Total RNA was extracted from the tissues using RNeasy Mini kit (Qiagen) according to the manufacturer's instructions including the DNAse treatment, and with the modification of adding a known, fixed amount of artificial RNA (Nurmi *et al.,* 2000b) to each sample after cell lysis. The tissue material stored in guanidine thiocyanate solution was first homogenized in the storage solution to include the RNA that had potentially dissolved into the storage buffer, after which the lysis buffer was added.

Extracted total RNA was characterized by gel electrophoresis for RNA degradation and the concentration was measured spectrophotometrically, after which RNA was reverse transcribed into cDNA using High Capacity cDNA Archive kit (Applied Biosystems) according to manufacturer's instructions and using the random primers provided in the kit. The cDNAs were stored at –20 °C.

4.3   Standards (I, II, III, IV)

A fixed amount of artificial RNA was used as an internal standard to track the inherent loss of RNA throughout the steps of the processing protocol, and target-specific, external DNA standards were used in real-time PCR.

4.3.1   *Internal RNA standard*

A mutated form of wild-type *KLK3* (the gene coding for the protein PSA) RNA was used throughout the study as the internal standard. Compared to the wild-type *KLK3* sequence, the standard, called mmPSA, contains a deletion and inversion in its sequence to make it an unrecognizable target for the *KLK3* real-time PCR assay. The standard used here had been manufactured earlier at the Department of Biotechnology, University of Turku (Nurmi *et al.,* 2000b). A known amount of mmPSA, 10^6 molecules, was added to each tissue and blood sample during RNA extraction. The amount of mmPSA present in sample cDNAs was measured by a separate mmPSA-specific real-time PCR assay to obtain a value for the extent of RNA loss during sample processing; this was subsequently used for normalizing the target mRNA copy numbers. A control for the real-time PCR assay, representing the maximum yield of internal standard RNA, was prepared by performing reverse transcription on the same amount of internal standard RNA as was added to the samples.

4.3.2   *External DNA standards*

Purified PCR products were used as external DNA standards in each real-time PCR assay. For each mRNA target, the corresponding PCR product was produced in an end-point PCR assay and the DNA was purified from agarose gel. The DNA concentration was measured with the fluorescein-based Picogreen reagent kit (Invitrogen), and the final product was diluted to different concentrations for use in real-time PCR assays.

4.4   Real-time PCR (I, II, III, IV)

The expression of the target genes (*BMP-6*, *FGF-8b*, *KLK2*, *KLK3*, *KLK4*, *KLK15*, *MSMB*, *PCA3*, *PSCA*, *SPINK1*, *TMPRSS2-ERG* III, *TMPRSS2-ERG* VI, *TRPM8*) was determined by real-time PCR assays. The assay principle is based on a closed-tube concept that utilizes oligonucleotide probes, and it was previously developed at the Department of Biotechnology, University of Turku (Nurmi *et al.,* 2002).

4.4.1   *Assay design*

Real-time PCR assays were specifically designed for each gene target and a small amplicon size was preferred to minimize the effect of RNA degradation on the results. The amplicon location was designed to preferably match the sequence between exon-intron boundaries in order to favor the amplification of reverse-transcription produced cDNA and avoid the amplification of traces of potential contaminating DNA in the samples. Previously published primers and probes were utilized for *PCA3* (de Kok *et al.*, 2002), *KLK2* (Ylikoski *et al.*, 2001; Rissanen *et al.*, 2007), *KLK3* (Nurmi *et al.*, 2000a; Nurmi *et al.*, 2002) and *TMPRSS2-ERG* III assays (Tomlins *et al.*, 2005; Cerveira *et al.*, 2006). The oligonucleotides used in the other mRNA assays were

designed specifically in this doctoral work. Assay performance was optimized individually for each assay by adjusting reaction conditions to maximize sensitivity and reproducibility, and to minimize unspecific amplification.

4.4.2   The principle of real-time detection of amplification products

The real-time detection principle of PCR amplification products is based on a previously published closed-tube concept (Nurmi *et al.,* 2002). Briefly, target-specific oligonucleotide probes hybridize to the target DNA sequence of the PCR product before the primers anneal (Figure 4).
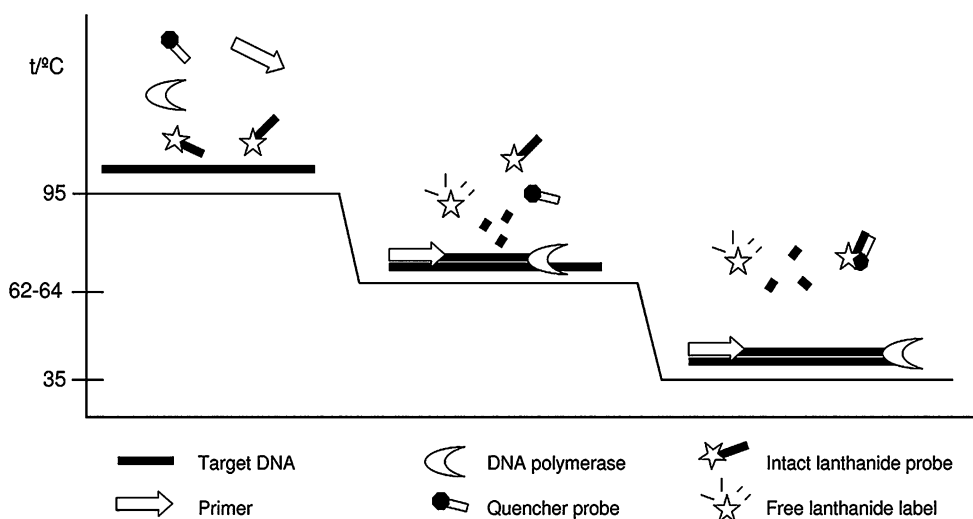
Figure 4. The principle of the real-time detection of PCR amplification. The accumulation of the PCR products was monitored in real-time during the PCR cycling using target-specific oligonucleotide probes labeled with lanthanide chelates and complementary probes carrying quencher molecules. (Figure from original publication **I**.)

At the annealing/extension temperature, DNA polymerase, while synthesizing the new DNA strand, encounters the hybridized probe and cleaves it off, simultaneously and irreversibly releasing the lanthanide chelate label from the 5' end of the oligonucleotide into the solution. The fluorescence of the intact probes is quenched by separate oligonucleotide probes carrying quencher molecules; these are capable of specifically hybridizing to intact reporter probes and turning off the fluorescence of their lanthanide chelate labels. Hence, fluorescence signal is recorded from released lanthanide chelate labels only and it is proportional to the amount of accumulated PCR products. The fluorescense is measured in a time-resolved manner to further reduce the background fluorescence.

4.4.3   Real-time PCR reagents and conditions

The sequences of the oligonucleotides used in the real-time PCR assays of this study are shown in Table 6. PCR primers, as well as quencher probes containing either QSY-7[®] or Dabcyl at the 3' end as the quencher molecule, were purchased from Thermo. Reporter probes were also purchased from Thermo, and they contained a phosphate group at the 3' end and an amino-C6 modification at the 5' end.

They were labeled in-house with terbium or europium chelates according to previously developed protocols where the lanthanide chelates were attached to the amino group (Nurmi *et al.,* 2000a). Briefly, target-specific oligonucleotides were incubated with a 100-fold molar excess of the lanthanide chelate overnight at +4 °C and subsequently purified with size-exclusion gel chromatography (Nick columns, Pharmacia) and high-performance liquid chromatography using a gradient between 0.05 M triethylamine acetate (TEAA) in H_2O (pH 6.8) and 0.05 M TEAA in acetonitrile (pH 6.8) as the eluting component. Purified oligonucleotides were quantified for DNA concentration and fluorescence, tested for quenching properties, and stored at –20 °C.

In each PCR reaction, 2.5 µL of cDNA or DNA standard were used as template on a 96-well plate (ABgene Thermo-Fast 96 Robotic Plate white, Thermo Scientific, or Hard-Shell PCR plate BLK/WHT, Bio-Rad). In negative control PCR reactions, 2.5 µL of DMPC-treated H_2O were used instead of DNA template. Total reaction volume was 25 µL in all assays except for the *TMPRSS2-ERG* III assay, where it was 10 µL. Hotmaster DNA polymerase (Eppendorf) was used in the assays for *KLK2*, *KLK3*, *PSCA,* and *TRPM8* mRNAs, and AmpliTaq® Gold DNA polymerase (Applied Biosystems) was used in all the other assays. Both polymerases contain 3'->5' exonuclease activity that is essential for the reporter probe cleavage. The detailed concentrations of the reagents in each assay are shown in Table 7.

The PCR thermal profile consisted of a denaturation step (15 s at +95 °C) and an annealing/extension step (1 min at +64 °C or +62 °C) that were repeated for 45 cycles in a PTC-200 thermal cycler (MJ Research). For added specificity, the annealing/extension was performed at the higher temperature, +64 °C, for the first ten cycles, and in the remaining cycles at +62 °C. Starting at cycle 11 for *TMPRSS2-ERG* III assay and at cycle 17 for all the other assays, the fluorescence signals were measured from the reaction wells in a time-resolved manner with a fluorometer (Victor, PerkinElmer) after every odd-numbered cycle. Measurement was carried out after a 15-second incubation at +35 °C. When AmpliTaq® Gold DNA polymerase was used, the thermal cycling began with an additional 10-minute enzyme activation step at +95 °C.

Table 6. The oligonucleotides used in the real-time PCR assays of this doctoral work.

Oligonucleotide	Sequence (5'->3')	Database sequence number	Position in sequence
BMP-6			
5' primer	GCA TCA TCA GCA CAG AGA CTC TGA C	NM001718	1025–1046
3' primer	ACG TGG ACT CCA TCC CTT G	NM001718	1177–1195
reporter probe	GGC TGG AAT TTG ACA TCA CGG CCT G	NM001718	1093–1117
quencher probe	TCA AAT TCC AGC C	NM001718	1093–1105
FGF-8b			
5' primer	CTG CCT CCA AGC CCA GGT A	NM_006119	224–242
3' primer	CGG CTG AGC TGA TCC GTC A	NM_006119	295–313
reporter probe	GCT CTG CTC CCT CAC ATG CTG TGT	NM_006119	267–290
quencher probe	ATG TGA GGG AGC AGA GC	NM_006119	274–290
KLK2			
5' primer	GAA CCA GAG GAG TTC TTG CG	NM005551	522–541
3' primer	CCC AGA ATC ACC CCC ACA A	NM005551	665–683
reporter probe	ACA TGT GTG CTA GAG CTT ACT CTG AGA AGG	NM005551	586–615
quencher probe	AGC TCT AGC ACA CAT GT	NM005551	586–602
KLK3			
5' primer	TGA ACC AGA GGA GTT CTT GAC	X05332	523–543
3' primer	CCC AGA ATC ACC CGA GCA G	X05332	667–685
reporter probe	CCT TCT GAG GGT GAA CTT GCG C	X05332	596–617
quencher probe	AAT CAC CCT CAG AAG G	X05332	600–601, 604–617
KLK4			
5' primer	GGC ACT GGT CAT GGA AAA CG	NM_004917	138–157
3' primer	CAG CCC GAT GGT GTA GGA GTT	NM_004917	223–243
reporter probe	CAG CAC CCA CTG CGG ATG CAC CAG	NM_004917	178–201
quencher probe	CCG CAG TGG GTG CTG	NM_004917	187–201

(continued from the previous page)

Oligonucleotide	Sequence (5'->3')	Database sequence number	Position in sequence
KLK15			
5' primer	CTG CCA AAG CCG CTT C	AY373373	186–201
3' primer	ATG ACC CGA GAC GTG G	AY373373	263–278
reporter probe	CGC CTG GGA GAG CAC AAC CTG	AY373373	211–231
quencher probe	CTC TCC CAG GCG	AY373373	211–223
mmPSA			
5' primer	TGA ACC AGA GGA GTT CTT GCA	X05332	523–543
3' primer	CCC AGA ATC ACC CGA GCG A	X05332	667–685
reporter probe	CCT TCT GAG GGT GAT TGC GCA C	X05332	594–601, 604–617
quencher probe	AAT CAC CCT CAG AAG G	X05332	600–601, 604–617
MSMB			
5' primer	CCT GCT TAT CAC AAT GAA TGT TC	NM_002443	20–42
3' primer	CAT TCA CTG ACA GAA CAG GTC	NM_002443	347–367
reporter probe	TCC TTC TTC TCC ACC ACG ATA TAC TTG C	NM_002443	310–337
quencher probe	GTG GAG AAG AAG GA	NM_002443	324–337
PCA3			
5' primer	GGT GGG AAG GAC CTG ATG ATA C	AF103907	95–116
3' primer	GGG CGA GGC TCA TCG AT	AF103907	505–521
reporter probe	AGA AAT GCC CGG CCG CCA TC	AF103907	478–497
quencher probe	CCG GGC ATT TCT	AF103907	478–489
PSCA			
5' primer	CTG TTG ATG GCA GGC TTG GC	NM005672	77–96
3' primer	GGC CAA CTG CGC GGA TG	NM005672	211–227
reporter probe	AGC CAG GCA CTG CCC TGC TGT G	NM005672	102–123
quencher probe	GCA GTG CCT GGC T	NM005672	102–114

(continued from the previous page)

Oligonucleotide	Sequence (5'->3')	Database sequence number	Position in sequence
SPINK1			
5' primer	GAC CTC TGG ACG CAG AAC	NM_003122	96–113
3' primer	GTA ACA TTT GGC CTC TCT TCC	NM_003122	199–219
reporter probe	AAG GTA ACA GGC ATC TTT CTT CTC AGT G	NM_003122	124–151
quencher probe	TGC CTG TTA CCT T	NM_003122	124–136
TMPRSS2-ERG III			
5' primer	TAG GCG CGA GCT AAG CAG GAG	NM_005656.3	4–24
3' primer	GTA GGC ACA CTC AAA CAA CGA CTG G	NM_004449.4	338–362
reporter probe	AGC GCG GCA GGA AGC CTT ATC AGT T	NM_005656.3 and NM_004449.4	57–64 and 310–326
quencher probe	TTC CTG CCG CGC T	NM_005656.3 and NM_004449.4	57–64 and 310–314
TMPRSS2-ERG VI			
5' primer	CGG CAG GTC ATA TTG AAC ATT CC	NM_005656.3	73–95
3' primer	GCA CAC TCA AAC AAC GAC TGG	NM_004449.4	338–358
reporter probe	CTT TGA ACT CAG AAG CCT TAT CAG TTG TGA	NM_005656.3 and NM_004449.4	139–149 and 312–330
quencher probe	GGC TTC TGA GTT CAA AG	NM_005656.3 and NM_004449.4	139–149 and 312–317
TRPM8			
5' primer	GAG TTG GAT CAA ATG GCT CAA	NM_024080	1165–1185
3' primer	GGT GCT GAA GGC TTT GTA TAG	NM_024080	1274–1294
reporter probe	ATG GCA TTG CTC ACA ATT TCA TCC CCA	NM_024080	1237–1263
quencher probe	GTG AGC AAT GCC AT	NM_024080	1250–1263

Table 7. The reagent concentrations in the real-time PCR assays.

Target mRNA	Primers (nmol/L)	Deoxyribo-nucleotides (mmol/L)	Polymerase (U/µL)	Added MgCl$_2$ (mmol/L)	Reporter probe (nmol/L)	Quencher probe (nmol/L)
BMP-6	100	0.2	0.025	2.5	4	170
FGF-8b	100	0.2	0.025	2.5	17	170
KLK2	100	0.2	0.016	-	17	170
KLK3	100	0.2	0.016	-	17	170
KLK4	100	0.2	0.025	2.5	17	170
KLK15	100	0.2	0.016	2.5	17	170
mmPSA	100	0.2	0.025	2.5	17	170
MSMB	100	0.1	0.025	2.5	17	170
PCA3	100	0.2	0.025	2.5	17	170
PSCA	100	0.2	0.016	-	17	170
SPINK1	100	0.1	0.025	2.5	17	170
TMPRSS2-ERG III	100	0.2	0.025	1.5	170	1700
TMPRSS2-ERG VI	500	0.4	0.050	2.5	43	170
TRPM8	100	0.2	0.016	-	17	170

4.5   Immunohistochemistry (IV)

The ERG protein expression was determined from the FFPE (formalin-fixed, paraffin-embedded) macro blocks of tissue in the prostate cross-section study (**IV**). Sections of 5 µm in thickness were cut from the blocks with a microtome and stained with a 1:250 dilution of the rabbit monoclonal ERG antibody (clone EPR3864, Epitomics). Detection was carried out with EnVision™+ Dual Link System-HRP (Dako) as the secondary antibody, and using DAB+ Chromogen (Dako) for the visualization of the stained tissue areas.

4.6   Data analysis

4.6.1   Real-time PCR data (I, II, III, IV)

Signal-to-background ratios were calculated for each reaction and plotted against the PCR cycle number. Quantification cycles (C_q) were determined visually for each

reaction as the cycle where the fluorescence level started to significantly differ from the background level. The differences between the C_qs of the external DNA standard reactions and the C_qs of the reactions of the maximum yield of internal standard RNA were calculated for each DNA standard concentration separately, and the standard plots were constructed by plotting the obtained differences in the C_qs against the ten-based logarithm of the target-specific standard concentration. The same difference in the C_qs was determined for each unknown sample reaction, and the mRNA copy numbers were calculated on the basis of that and normalized to sample size using either volume or total RNA amount. After normalization, the blood sample data are presented as mRNA copies per mL of blood, and the tissue sample data as mRNA copies per μg of total RNA.

Samples were run as three PCR replicate reactions and, unless stated otherwise, were considered to contain the target only when all three reactions produced fluorescence signals that differed from the background.

The limit of detection (LOD) was defined as the lowest concentration of external DNA standards that produced a fluorescence signal differing from the background. The limit of quantification (LOQ) was defined for the blood samples as the concentration of mRNA in blood that consistently produced the above-mentioned level of fluorescence (**I**).

4.6.2   Immunohistochemistry data (IV)

The tissue slides stained with the ERG antibody in the immunohistochemistry experiments were evaluated for ERG expression by a pathologist. For a comparison of the locations of positive staining with the corresponding tissue samples in the mRNA experiments of the original manuscript **IV**, digital images were taken of the tissue slides used in the immunohistochemistry experiments, and divided into an equal amount of regions similarly to the tissue cross-sections, and each region being marked as either containing detectable ERG expression or not.

4.6.3   Statistical analysis

Statistical analysis for publications **II** and **III** was performed with Stata 10.0 at the Department of Epidemiology and Biostatistics of Memorial Sloan-Kettering Cancer Center (New York, NY, USA). Univariate logistic regression was used to analyze the association between mRNA expression and the studied parameters of the tissue samples.

SPSS 20.0 (IBM) was used to perform the statistical analyses for publication **IV**, and nonparametric tests were used to analyze the associations between histology and mRNA expression levels.

# 5 SUMMARY OF RESULTS AND DISCUSSION

This doctoral work concentrated on evaluating the RNA expression of 12 candidate biomarker genes in prostate tissue, but it also assessed the possibility to detect two of them in circulating cells in blood. A summary of the results of the original publications is presented here.

5.1   Gene expression in circulating tumor cells in peripheral blood (I)

The current diagnostic routine of prostate cancer ultimately relies on the histological analysis of prostate biopsies. The biopsy procedure is an invasive method that may also be risky and have adverse effects such as bleeding or infection, and will prove inconvenient for monitoring purposes when frequent follow-up sampling is required. For clinical diagnostics, less invasive methods such as urine or blood tests would therefore be preferred.

Cells can be shed into blood circulation by the primary tumor in the prostate due to cancer-related changes in the organ structure even when the tumor foci are small or the volume of the tumor is low (Schmidt *et al.,* 2006a; Ali *et al.,* 2010). These circulating tumor cells are thought to be the cause of metastatic forms of cancer if they migrate to a distant secondary site, attach and start proliferating. The presence of circulating tumor cells has been particularly suggested as a monitoring tool to predict potential cancer recurrence. Using varied methods, the tumor cells can be detected in blood either on the basis of their cancer-specific or their organ-specific characteristics, such as particular nucleic acid content, but the challenge is their low level in blood. The high background count of lymphocytes makes it difficult to reliably analyze the tumor-related cell content of blood, and sensitive analysis techniques, such as RT-PCR, are therefore required (Alix-Panabieres & Pantel 2013).

In this doctoral work, two candidate RNAs, those of *PCA3* and *KLK3,* were evaluated for their suitability for the detection of circulating prostate cancer cells in blood. The RNA expression of these genes was measured in peripheral blood samples of 67 prostate cancer patients and 24 noncancer controls with real-time qRT-PCR assays. Measurable levels of either of the studied RNAs were only found in patients with metastatic prostate cancer. Samples from two out of the nine studied men with metastasized prostate cancer contained measurable levels of *PCA3* RNA (Figure 5A), but *PCA3* RNA transcripts were not detected in any of the samples from healthy controls (n=16), men with localized prostate cancer (n=58), or men with other prostatic diseases (n=8). Quantifiable levels of *KLK3* mRNA were found in 4/9 blood samples of men with metastatic prostate cancer (Figure 5B).
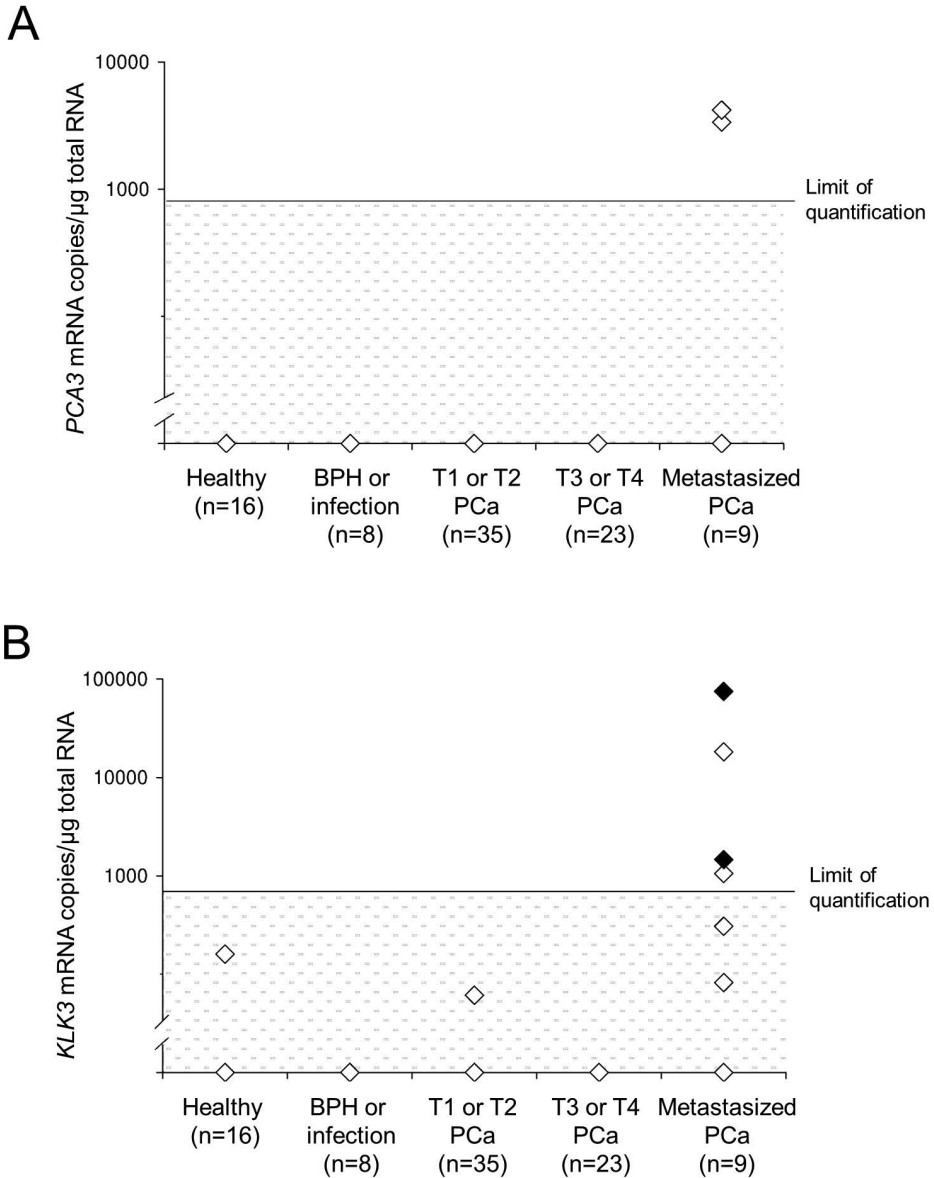
Figure 5. *PCA3* and *KLK3* mRNA expression in the blood of prostate cancer patients, men with other prostatic diseases and healthy individuals. BPH, benign prostatic hyperplasia; PCa, prostate cancer. A. *PCA3* mRNA expression levels. B. *KLK3* mRNA expression levels. Open diamonds show samples with only *KLK3* mRNA expression, closed diamonds denote samples that also contained *PCA3* mRNA. (Figure modified from original publication **I**.)

Additionally, one healthy control, one sample from a man with clinically localized prostate cancer and two samples from the metastatic group were discovered to contain *KLK3* mRNA, but at levels lower than the assay LOQ, making them, therefore, unquantifiable. The low overall number of *PCA3* or *KLK3* positive samples in this study could be explained by the absence of prostate cells in the samples that were mostly obtained from cases of localized cancer. The even lower positivity rate of *PCA3* compared to *KLK3* could also be due to a lower expression level of *PCA3*. This was supported by the finding that the median mRNA expression of *KLK3* was 28 times higher than the *PCA3* RNA level in prostate tissue of ten tested radical prostatectomy specimens.

The concentration of circulating tumor cells is usually very low in blood compared to the background of millions of blood cells, and the current consensus is that an enrichment step is needed to improve the detection sensitivity. Enriching can be accomplished by capturing the tumor cells from blood with immunoaffinity techniques, using their tumor cell-specific surface molecules as markers, or by using their physical properties such as size or electric charge as the factors separating them from peripheral blood cells (Alix-Panabieres & Pantel 2013). In this work the resources were not available to perform the enrichment, but a strong concordance has been demonstrated for the performance of this RT-PCR concept and the FDA-cleared enrichment method, CellSearch® (Veridex), to detect circulating prostatic tumor cells in blood (Helo *et al.,* 2009). Additionally, results similar to those obtained in this work were also obtained in another study even after using immunomagnetic enrichment of the circulating tumor cells: *PCA3* RNA was found only in the blood of men with advanced prostate cancer with no *PCA3* RNA detected in healthy controls or men with androgen-dependent cancer (Jost *et al.,* 2010). Also the observed difference of approximately one magnitude between *KLK3* and *PCA3* RNA levels was in concordance with the results of this doctoral work. However, another report using enrichment techniques on blood from men with CRPC, found *PCA3* RNA in the blood circulation of all 15 study subjects, and also low levels in one of the healthy controls (Shaw *et al.,* 2008). Other reports on *PCA3* RNA in men are rare but some studies have detected it even without the enrichment of the cells (Marangoni *et al.,* 2006; Neves *et al.,* 2013). The assays performed in this doctoral work did not detect any *PCA3* expression in the healthy individuals.

Findings of *KLK3* mRNA in peripheral blood have been more frequent than those of *PCA3* RNA, and several qualitative and quantitative methods have been employed for detection. Already in 1994, the detection of *KLK3* mRNA in blood of men with localized prostate cancer was reported, and it was found with even higher frequency in metastatic cases (Katz *et al.,* 1994). As noted also in this doctoral work, the trend is to find *KLK3* mRNA more frequently in prostate cancer patients with a non-organ-confined disease (Olsson *et al.,* 1996; Zhang *et al.,* 2008), but as reviewed by Kalfazade and others, the data on whether the preoperative levels of *KLK3* mRNA in blood help in staging the cancer or predicting recurrence are contradictory (Kalfazade

et al., 2009). Most of the early data are obtained by qualitative or semiquantitative methods, which can account for the discrepancy between different results, and qRT-PCR is recommended for the most accurate information (Kurek *et al.,* 2003).

5.2   Gene expression in prostate tissue (II, III, IV)

While circulating tumor cells provide an interesting, a less invasive possibility for prostate cancer detection and monitoring, the research for finding and validating new biomarkers starts often at tissue level. The potential cancer-related cells that are shed into the blood circulation originate from the primary organ, and therefore the molecular examination of prostate tissue is highly important. Furthermore, the application of molecular assays on biopsy material could add value to the histological analyses at diagnosis.

Radical prostatectomy is a routine procedure in prostate cancer treatment, and tissues from radical prostatectomy specimens are rather readily available for research, providing both cancerous and histologically benign samples. Noncancerous tissue is more challenging to obtain due to the limited availability of suitable candidates. Benign hyperplastic prostate tissue collected at TURP procedures is often used as control tissue in studies. In this doctoral work, the gene expression of the candidate biomarkers in prostate tissue was examined in radical prostatectomy specimens and in cystoprostatectomy specimens from men with urinary bladder cancer. Cystoprostatectomy comprises the removal of prostate along with the bladder and a subsequent pathological dissection of the prostate for incidental tumors; this is an advantage compared to the material obtained from TURP procedures, where only tissue shavings are removed and collected, but the remaining organ can still harbor unknown malignancies.

The scientific literature offers a continuous flow of new candidate genes for biomarkers of prostate cancer and 12 such genes were selected for this doctoral work: *BMP-6*, *FGF-8b*, *KLK2*, *KLK3*, *KLK4*, *KLK15*, *MSMB*, *PCA3*, *PSCA*, *SPINK1*, *TMPRSS2-ERG* and *TRPM8*. The selection was made on the basis of their altered expression in prostate cancer at protein or RNA level in previously reported studies, and the RNA expression of these genes was measured in prostate tissues of 87 men with clinical prostate cancer. The expression of *KLK3*, *PCA3*, *SPINK1,* and *TMPRSS2-ERG* was also measured in further 19 men with bladder cancer but without clinical prostate cancer. To be able to assess the differences in gene expression with regard to cancer development, samples were collected from both cancerous and histologically benign areas of the radical prostatectomy specimens of prostate cancer patients. The measured RNA levels were compared to sample characteristics, such as sample histology, Gleason grade, and the pathological stage of the cancer, to find out which genes performed best at stratifying between cancerous and benign tissue or between different stages of cancer.

KLK2, *KLK3*, *KLK4* and *MSMB* mRNAs were found to be expressed generally at the highest levels, followed by *TRPM8*, *BMP-6*, *PCA3*, *PSCA*, and *SPINK1* RNAs (Figure 6) (**II**, **III**). The expression levels for *KLK15*, *TMPRSS2-ERG* and *FGF-8b* mRNAs were the lowest among the studied genes and in fact, *FGF-8b* mRNA was undetectable in 70% of the radical prostatectomy samples. Of the two studied mRNA variants of *TMPRSS2-ERG* fusion gene, isoform III was detected in 48% and isoform VI in 47% of the samples of the radical prostatectomy cohort.
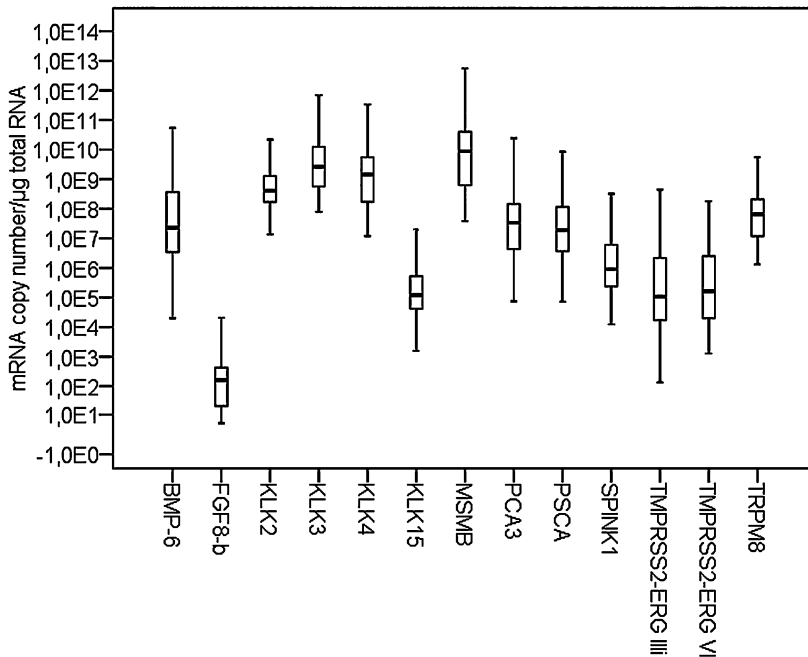
Figure 6. Expression levels of 13 target genes in 174 prostate tissues from prostate cancer patients. Levels are shown only for those samples where expression was quantifiable. The 10/25/50/75/90th percentiles are marked in the figure.

5.2.1   *PCA3 (II, III, IV)*

PCA3 gene was first found to be associated with prostate cancer in 1999 (Bussemakers *et al.,* 1999). Its overexpression in cancerous tissue has been reported several times and depending on the study, the magnitude has ranged from 10 up to 100-fold overexpression (Bussemakers *et al.,* 1999; de Kok *et al.,* 2002; Hessels *et al.,* 2003; Robert *et al.,* 2013). In this doctoral work a statistically significant, 5.8 times higher expression was seen in the carcinoma areas of 86 radical prostatectomy samples when compared to the median expression in the 88 samples from the histologically benign areas of the same cohort (p<0.05) (Figure 7) (**II**). This was at the lower end of the reported ranges, but a more pronounced, over 600-fold increase in *PCA3* RNA

expression was found when the cancerous tissues were compared to the 19 cystoprostatectomy tissues of men without clinical cancer (p<0.0001) (Figure 7) (**III**). The resulting, over 100-fold difference in the medians between *PCA3* RNA levels in the histologically benign prostate tissue of cancerous prostates and prostates without clinical cancer suggests that the carcinoma-surrounding, but histologically benign, areas of the cancerous prostates already show molecular changes, i.e. increased *PCA3* expression.
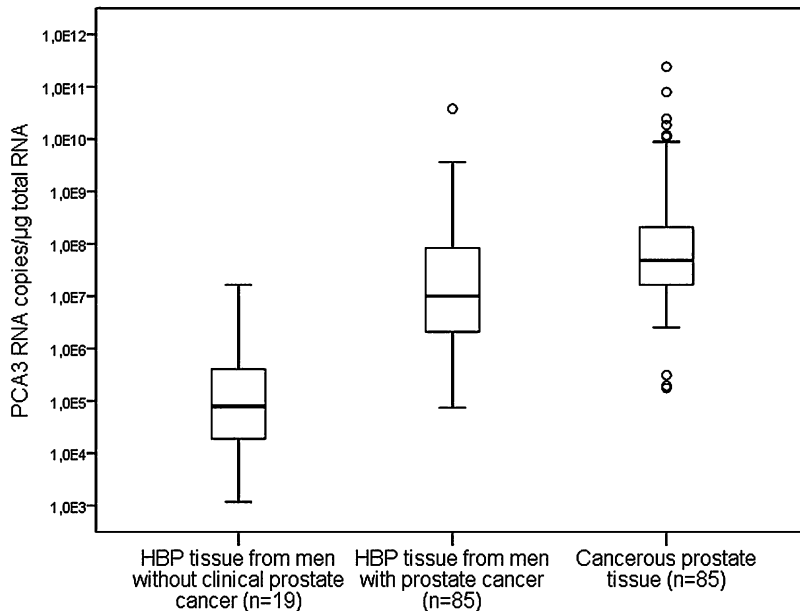
Figure 7. Expression of *PCA3* RNA in the histologically benign tissue of prostates without clinical prostate cancer, prostates with malignant tumor, and cancerous tissue. Levels are shown only for those samples where expression was quantifiable. The 10/25/50/75/90th percentiles are marked in the figure and the open circles denote the outlier values. HBP, histologically benign prostate.

The molecular level alterations in gene expression can be explained by the field cancerization theory. First described in 1953 (Slaughter *et al.,* 1953), it suggests that a larger area of the tissue than just the area covering the tumor foci is originally changed in terms of molecular content, and that a certain area within that zone can then further develop into a neoplastic lesion after experiencing the additional necessary changes in cell functions. The altered expression of *PCA3* in benign areas of cancerous prostates has previously been studied by *in situ* hybridization (Popa *et al.,* 2007), and a finding that benign glands in closer proximity to the carcinoma show *PCA3* expression more frequently than similar glands more distant from the malignant tumor, was claimed to be caused by the cancer field effect. Approximately 30% of the benign glands were

demonstrated to be positive for *PCA3* RNA. More recently, Warrick and colleagues (2014) found *PCA3* RNA expression in benign glands of nine cases out of the 41 studied prostatectomy specimens. Yet, neither of these studies found the phenomenon of field cancerization regarding *PCA3* to be as widespread as the results of this doctoral work would suggest. The question of the histologically benign samples being contaminated by cancer cells or their nucleic acids could therefore be raised here, but several precautions were taken to avoid crosscontamination in this doctoral work. Sterile, disposable laboratory equipment was always used and the handling of clean PCR reagents was carried out in a designated hood that was physically separate from the workspaces dedicated to sample processing, RNA extraction, and PCR template work. The workspaces were cleaned with ethanol and ultraviolet radiation after every use to deplete and destroy any leftover nucleic acids. The opening of reagent tubes post-PCR amplification that was required by the gel electrophoresis experiments, could have naturally posed a threat of crosscontamination by PCR products, and therefore it was also performed in a different laboratory room. Negative control reactions with sterile water as template were always run in parallel to every real-time PCR assay to ensure that the reagents and disposables were free of contaminating DNA.

The origins of the benign tissues used for controls in previous studies are not always specified, but Hessels and colleagues (2003) used a study set-up similar to the one used in this doctoral work, comparing *PCA3* RNA levels between carcinoma tissues and benign areas in cancerous prostates. They reported a 10–60-fold overexpression which is still of a higher magnitude than was found in this doctoral work even though there is an equal possibility that both cohorts could have been affected by the cancer field effect. Robert and colleagues (2013) have, however, shown increased *PCA3* RNA levels also in the benign regions of cancerous prostates when they were compared to BPH tissue. The *PCA3* expression was found to be three times lower in the BPH tissue.

Of the cohort of 87 prostate cancer patients, 46 men provided one cancerous and one histologically benign sample with quantifiable *PCA3* expression, enabling the examination of the RNA level variation between matched samples. The median overexpression observed in the cancerous areas compared to the matched benign area was 2.9-fold (**II**). The hypothesis of altered expression in the histologically benign regions was further studied by obtaining cross-sections from five prostates with carcinoma, systematically dividing the tissue slice into smaller areas each covering 5x5 mm of tissue, and measuring the *PCA3* expression in all of those areas separately (**IV**). The adjacent superior and inferior tissue was examined histologically by a pathologist, providing information about the matched morphology of the tissue, and on the basis of these data, each sample area was assigned as a carcinoma area, a PIN area, or a histologically benign area. The carcinoma areas were found to contain 3.1–3.6 times higher levels of *PCA3* RNA than the benign areas (p<0.001), depending on whether the benign sample was located right next to a carcinoma sample – which admittedly, for technical reasons, could therefore contain occasional cancer cells – or further away

from the malignant tumor (Figure 8). This value supports the finding of 2.9-fold difference (**II**) that was obtained using just two matched samples per prostate.
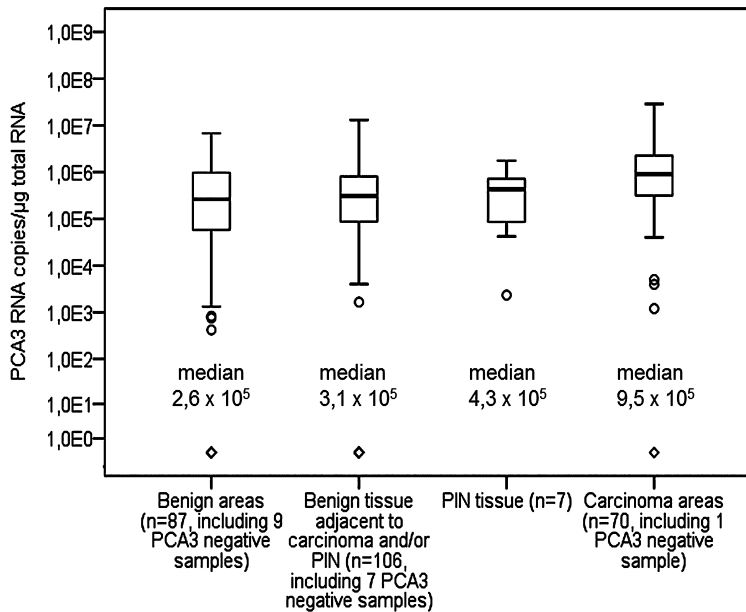
Figure 8. *PCA3* expression in the benign, malignant and PIN lesion areas of cross-sections of five cancerous prostates. The 10/25/50/75/90th percentiles are marked in the figure and the open circles denote the outlier values. Open diamonds denote the negative samples. PIN, prostatic epithelial neoplasia. (Figure modified from original manuscript **IV**.)

The levels of *PCA3* were found to be positively associated with advanced cancer stage with higher *PCA3* levels in the tissues of men with stage T3 or T4 cancer ($p=0.015$), but no association was seen between the Gleason grade of the tissue and the *PCA3* RNA level. This suggests that *PCA3* alone cannot provide a reliable cancer staging.

The fact that an increase in *PCA3* RNA expression is seen already in the histologically benign areas of a cancerous prostate when compared to a clinically noncancerous prostate, could indicate a possibility to identify cancerous prostates on the basis of samples obtained from their histologically benign areas. There was an overlap in RNA levels between the two groups of histologically benign samples but, if validated, this finding suggests that this molecular assay could potentially provide further useful information in addition to the histological analysis of the tissue for example for men with negative prostate biopsies. The current prostate biopsy sampling protocol cannot, despite ultrasound guidance, guarantee that the biopsy needle will reach the tumor focus and, therefore, the assay would be a valuable addition to assessing the risk of

cancer in the prostate, if relevant molecular information could be retrieved also from those biopsy cores that missed the carcinoma.

5.2.2   TMPRSS2-ERG (III, IV)

TMPRSS2-ERG fusion gene was discovered in 2005 and it is thus far the most common genetic rearrangement seen in epithelial cancers (Tomlins *et al.,* 2005). Depending on the study cohort, the gene fusion can be detected in 40–70% of prostate cancers, but its significance or that of its mRNA variants in terms of patient outcome is still being debated. In this doctoral work, *TMPRSS2-ERG* mRNAs were found to be expressed in the prostates of 72% of the 87 men with prostate cancer, which is in accordance with the previous reports. Two of the over 20 reported mRNA isoforms (Tomlins *et al.,* 2009) produced by the different variants of the fusion gene were selected for the study: isoform III is the most commonly found variant (Tomlins *et al.,* 2005) and isoform VI was associated with an aggressive form of prostate cancer in one of the early studies (Wang *et al.,* 2006).

Both mRNA variants, *TMPRSS2-ERG* III and *TMPRSS2-ERG* VI, were found more frequently in the cancerous tissues of men with prostate cancer than in the histologically benign areas of the same patient cohort (p=0.022 and 0.026, respectively) (**III**). Of the 86 cancerous tissue samples, 49 (57%) showed expression of isoform III and 47 (55%) contained mRNA variant VI (Table 8). In the histologically benign tissues, *TMPRSS2-ERG* III was found in 35/88 (40%) samples and *TMPRSS2-ERG* VI in 34/88 (39%) samples. However, it has to be noted that among the samples where the fusion gene mRNA levels were quantifiable, cancerous tissue did not show significantly higher or lower *TMPRSS2-ERG* mRNA levels in comparison to the benign cohort.

In a notable contrast to the men with prostate cancer, only two in the group of 19 men without any indication of clinical prostate cancer showed a detectable expression of *TMPRSS2-ERG* in their prostate tissue (Table 8). Furthermore, both of those two prostates were later on found to contain incidental microscopic tumor foci elsewhere in the prostate. This finding gave rise to the systematic study of five prostate cross-sections (**IV**), where it was shown that even though the entire cross-section of a prostate obtained from a *TMPRSS2-ERG* fusion-positive cancer case was not expressing the fusion gene mRNA, the mRNA was, in addition to being expressed in the carcinoma regions and areas immediately adjacent to them, also detectable in five of the samples from the histologically benign tissue areas. However, these data can be considered as preliminary at best, due to the limited sample size. ERG protein expression was examined in the immediately adjacent cross-sections of tissue from the same prostates by immunohistochemistry, and positive ERG staining was limited to one prostate and to those carcinoma areas, where also *TMPRSS2-ERG* mRNAs were detected. However, despite the positive mRNA expression, additional areas where

ERG protein expression could not be detected were found, suggesting that the qRT-PCR assay has a higher sensitivity compared to the immunohistochemistry method.

Table 8. *TMPRSS2-ERG* III and VI mRNA expression detected in the prostate tissue of men with or without clinical prostate cancer. (Table modified from original publication **III**.)

	Number of samples				
Sample group	**Only *TMPRSS2-ERG* III mRNA detected**	**Only *TMPRSS2-ERG* VI mRNA detected**	**Both *TMPRSS2-ERG* III and VI mRNAs detected**	**Neither *TMPRSS2-ERG* III nor VI mRNA detected**	**Total**
Cancerous tissue from RP specimens	10 (12%)	8 (9%)	39 (45%)	20 (34%)	86
HBP tissue from RP specimens	11 (13%)	10 (11%)	24 (27%)	43 (49%)	88
HBP tissue from CP specimens	0 (0%)	1 (5%)	1 (5%)	17 (89%)	19

RP, radical prostatectomy; HBP, histologically benign prostate; CP, cystoprostatectomy.

Previous studies identifying *TMPRSS2-ERG* fusion gene areas with the same ERG-specific antibody that was used in this doctoral work had similar levels of correlations between the nucleic acid tests and immunohistochemistry. A study by van Leenders and colleagues (2011) showed that all samples that were found to be positive for *TMPRSS2-ERG* mRNA by qRT-PCR, also contained ERG protein, whereas Park and colleagues (2010) found three cases out of 131 cancers where *TMPRSS2-ERG* fusion was detected by FISH without corresponding ERG protein expression. Nineteen men with prostate cancer provided two cancerous radical prostatectomy samples for the study, and three (16%) of them showed the expression of *TMPRSS2-ERG* mRNAs in only one of their cancerous samples, suggesting that not all carcinoma foci within a single prostate contain the fusion gene, and indicating the multiclonal nature of the disease (**III**). It has also been shown that within a single prostate the malignant tumor can be *TMPRSS2-ERG* fusion-negative, and the benign area can present the fusion gene (Clark *et al.,* 2007). We found this to be rare, but possible: in six (13%) of the 48 cancer cases that provided one cancerous and one histologically benign tissue sample for this study, *TMPRSS2-ERG* mRNA expression was detected in only the histologically benign tissue and not in the matched cancerous sample. In 33/48 (69%) cases the *TMPRSS2-ERG* expression was concordant regarding the presence or absence of the fusion mRNA.

Furthermore, 24 (27%) and 39 (45%) of the 88 histologically benign and 86 cancerous radical prostatectomy samples, respectively, showed expression of both isoforms in the same sample (Figure 9). A particular tumor focus can contain more than one isoform of the gene fusion (Furusato *et al.,* 2008) and they can presumably be a result of differential mRNA splicing of a single DNA-level gene fusion (Clark *et al.,* 2007).
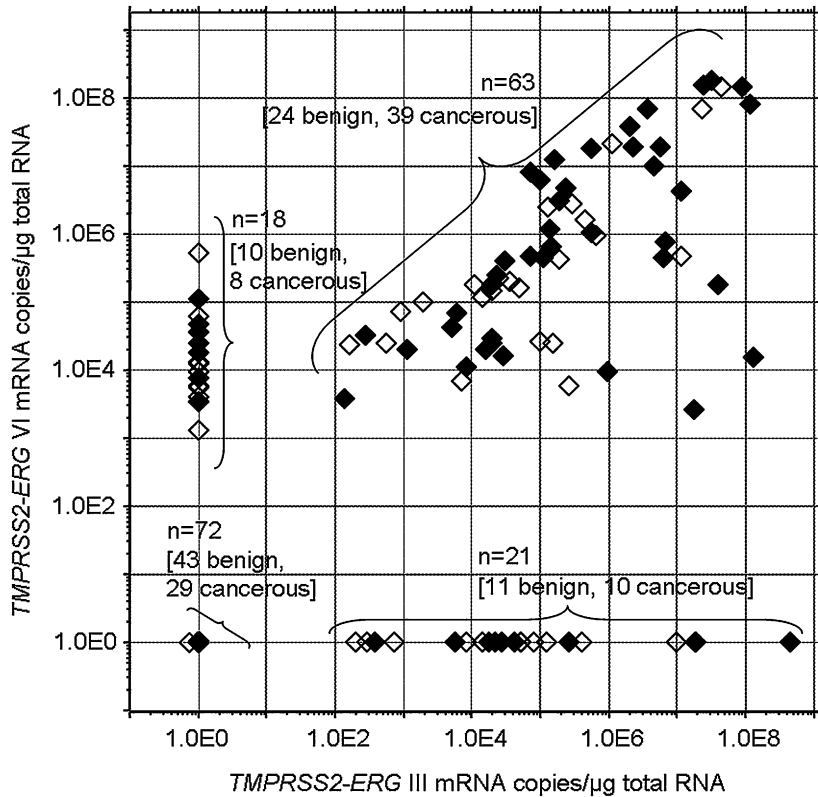
Figure 9. *TMPRSS2-ERG* III and VI mRNA expression in 86 cancerous and 88 histologically benign prostate tissue samples of radical prostatectomy specimens. Closed diamonds denote the cancerous samples and open diamonds represent samples from histologically benign areas. (Figure from original publication **III**.)

The claimed cancer specificity of the *TMPRSS2-ERG* fusion gene originally referred to carcinoma areas (Tomlins *et al.,* 2005), but later, there were reports stating that cancer-adjacent tissue, PIN lesions, and hyperplastic areas sometimes manifested *TMPRSS2-ERG* fusion (Clark *et al.,* 2007; Furusato *et al.,* 2008; Robert *et al.,* 2013). Still, so far, neither this doctoral work nor other studies have been able to find the fusion gene in the tissue samples of men without any prostatic diseases. A urine test for *TMPRSS2-ERG* has been positive in two men with negative prostate biopsies, but they could have had undetected cancers (Hessels *et al.,* 2007).

As described in section 5.2.1 discussing *PCA3* mRNA, the expression of *TMPRSS2-ERG* mRNAs in the histologically noncancerous areas can be due to molecular precursor changes in the tissue, and could provide the potential to use the phenomenon for identifying prostates currently harboring malignant foci, or running the risk of developing a carcinoma in the future. Furthermore, if a certain mRNA variant could be associated with an aggressive form of the disease, an early discovery could enable an early intervention with necessary measures.

5.2.3   PSCA (III)

PSCA mRNA was found to be underexpressed in the cancerous tissues of men with prostate cancer. The median expression level of *PSCA* was 2.9 times lower in them than in the histologically benign tissue of the same cohort (p=0.046). *PSCA* mRNA levels were not associated with the Gleason grade of the tissue or the advanced cancer stage. The overlap of the ranges of *PSCA* mRNA expression levels between cancerous and histologically benign areas and the barely statistically significant difference between the medians in the group indicate only a moderate trend of *PSCA* underexpression in cancer cells. The earliest reports on *PSCA* claimed increased expression in cancer detected by *in situ* hybridization (Zhigang & Wenlv 2004), but later, no significant difference was seen in mRNA levels between cancerous tissues and matched histologically benign samples when measuring them with qPCR (Schmidt *et al.,* 2006b). A protein level study examined differences between incidental cancers found in cystoprostatectomies and cancerous areas of radical prostatectomies, and despite the lower number of PSCA-positive cells in the incidentally found cancers, the difference was not statistically significant (Mazzucchelli *et al.,* 2009). In this doctoral work, the cystoprostatectomy specimens were not examined for *PSCA* expression due to a limited availability of material.

5.2.4   SPINK1 (III)

SPINK1 gene has been claimed to be overexpressed in prostate cancer and the expression has often been found to be limited to prostate cancer cases that do not present the *TMPRSS2-ERG* gene fusion (Tomlins *et al.,* 2008). In this doctoral work, *SPINK1* mRNA levels were indeed 2.9 times higher in the cancerous than in the histologically benign areas of cancerous prostates (p=0.047) (Figure 10). The lowest *SPINK1* mRNA levels were detected in the tissues of men without clinical prostate cancer – 10 times lower than in cancerous tissues (p<0.001). This led also to a statistically significant, 3.4-fold difference (p=0.010) in the median levels of *SPINK1* mRNA expression in histologically benign tissues depending on whether the tissue originated in a cancerous prostate or in a prostate without clinical cancer.
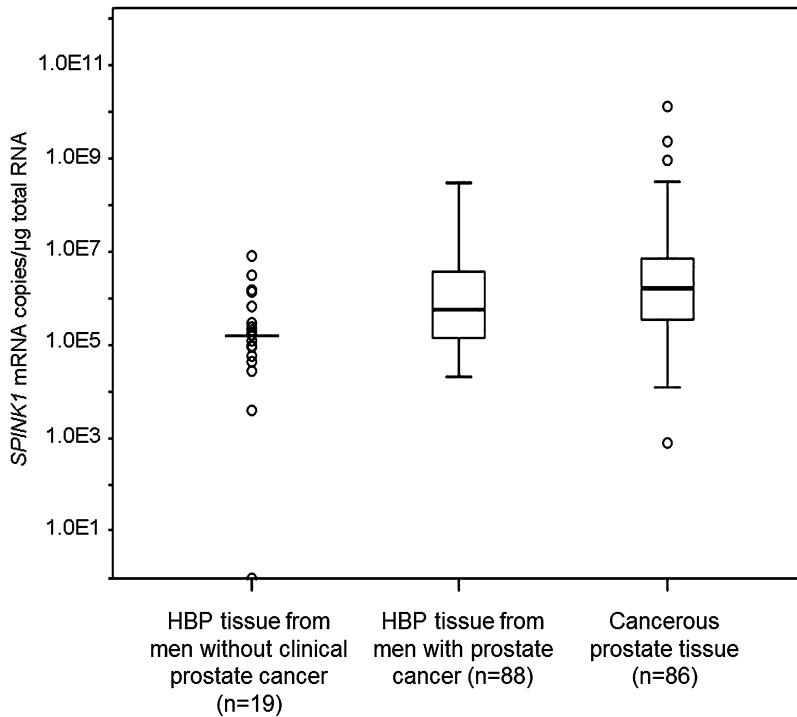
Figure 10. *SPINK1* mRNA expression in histologically benign tissues from men without and with clinical prostate cancer, and cancerous prostate tissue. For the group without cancer, the horizontal line denotes the median value, and the open circles denote *SPINK1* mRNA values in individual samples. For the other two groups, the 10/25/50/75/90th percentiles are marked in the figure and the open circles denote the outlier values. HBP, histologically benign prostate. (Figure modified from original publication **III**.)

In general, reports on quantifying *SPINK1* mRNA levels in prostate tissue are rare, as most of the previous data on prostate-related *SPINK1* expression are from immunohistochemistry or *in situ* hybridization experiments, or urine sample cohorts. Sabaliauskaite and colleagues (2012) determined *SPINK1* mRNA levels in a set-up similar to the cohort of this doctoral work, comparing cancerous and noncancerous tissues of 179 PCa patients, but did not find a significant difference between the groups. The levels in that study were normalized to a reference gene, which introduces a potential source of discrepancy between that data and the levels measured in this doctoral work, where a normalizing procedure towards an artificial, internal RNA control was used. However, it must be noticed that *SPINK1* mRNA expression was found in noncancerous areas also in the Sabaliauskaite study. In addition, Laxman and colleagues (2008) measured *SPINK1* mRNA levels in urine samples, and reported an increase in men with prostate cancer, but also saw a detectable *SPINK1* mRNA expression in men with negative biopsies.

In this work, *SPINK1* expression was neither found to be associated with the Gleason grade of the prostate tissue nor an advanced pathological stage of the cancer. Follow-up times were not long enough to enable a proper analysis of prognosis here, but higher *SPINK1* levels have been suggested to lead to poorer survival (Leinonen *et al.,* 2010).

SPINK1 mRNA expression and the mere presence of *TMPRSS2-ERG* fusion gene were not found to be correlated here, but in those men who had the fusion gene, the levels of *SPINK1* and *TMPRSS2-ERG* mRNA were statistically significantly associated (p=0.001–0.041, depending on the mRNA variant of *TMPRSS2-ERG*). A study where all *SPINK1* positive cancers were *TMPRSS2-ERG* negative (Tomlins *et al.,* 2008), used immunohistochemistry to detect TATI, the protein coded by *SPINK1*, and FISH to determine the presence of the fusion gene, as opposed to the qRT-PCR assays used in this work. Compared to the protein experiments, the highly sensitive qRT-PCR method could therefore detect lower levels of *SPINK1* expression, and conceivably there could also be differences between the transcription and translation levels of the gene. Nevertheless, another study also using FISH and immunohistochemistry for *TMPRSS2-ERG* and *SPINK1* level determinations in tissues of a hormonally-treated prostate cancer patient cohort did not find a mutual exclusivity between *SPINK1* and the *TMPRSS2-ERG* fusion either (Leinonen *et al.,* 2010). Instead, Leinonen and colleagues found an equal percentage (11% or 12%) of the fusion-negative and fusion-positive cases to contain the protein level expression of *SPINK1*. In the above-mentioned study of mRNA-level quantification in prostate tissue by Sabaliauskaite and colleagues (2012), an increase in *SPINK1* levels was seen in the *TMPRSS2-ERG* fusion-negative cases, but the expression was detectable in fusion-positive patients as well. Different criteria for categorizing samples as *SPINK1*-positive could also play a role in the contradictory reports via a varying interpretation of the immunohistochemistry staining data. The grading of staining intensities and the level of staining required for a positive observation are often independently decided by each research group.

5.2.5   KLKs 2, 3, 4, and 15 (II, III, IV)

The expression levels of *KLK3*, the gene coding for the PSA protein, and *KLK2* were among the highest target gene levels seen in the prostate tissue (Figure 6). However, no statistically significant differences were found between the tissues originating from a cancerous or histologically benign area of a prostate for either of these two or the kallikrein-related peptidase genes 4 or 15. Both *KLK2* and *KLK4* mRNAs are claimed to be overexpressed in prostate tissue in cancerous areas compared to benign hyperplastic tissue or normal glands (Herrala *et al.,* 2001; Xi 2004; Neves *et al.,* 2008; Avgeris *et al.,* 2011), but in this work no such overexpression was observed.

KLK3 mRNA levels were measured mainly for control purposes. *KLK3* expression was also measured in all of the samples covering the entire prostate cross-sections of the study described in original manuscript **IV**, but in that study, no significant differences

were found in *KLK3* mRNA levels that would have corresponded with the tissue histology. However, histologically benign tissue from men without clinical prostate cancer showed slightly lower levels of *KLK3* mRNA than tissues from cancerous prostates (1.9-fold difference in medians, p=0.032). This is in contrast to previous reports on *KLK3* mRNA levels being reduced in cancer compared to benign prostate tissue (Herrala *et al.,* 2001; Meng *et al.,* 2002). Nevertheless, as the only studied human kallikrein gene, *KLK3* was associated with the Gleason grade of the sample and the *KLK3* mRNA levels were, in fact, lower in tissues with Gleason grades 4 and 5 when compared to grades 2 and 3 (p=0.018).

KLK15 was the only human kallikrein gene in the study that was associated with advanced pathological stage (**II**). Tissues of men with cancer of the pathological stage 3 and 4 had statistically significantly higher levels of *KLK15* mRNA (p=0.032), and this finding was concordant with previous studies (Rabien *et al.,* 2010; Mavridis *et al.,* 2013). However, those studies reported also an increase in *KLK15* levels in cancerous tissue when compared to matched histologically benign glands or benign hyperplastic prostate tissue. An earlier study had examined the differential expression of both the classical mRNA variant of *KLK15* and the three alternatively spliced mRNA variants (Mavridis *et al.,* 2010), and only the classical variant was shown to be overexpressed in cancer. That is the form later associated with the pathological stage (Mavridis *et al.,* 2013). In this doctoral work, no distinction was made between the variants due to the fact that the information on their potential independent significance was not available at the time of the assay design. Therefore, the amplicon region of the *KLK15* assay extending from the junction of exons 2 and 3 downstream into exon 3, was designed to comprise a fragment of the sequence that is present in all reported isoforms of *KLK15* mRNA (Kurlender *et al.,* 2005). Rabien and colleagues do not state explicitly which variant they analyzed, but on the basis of the amplicon sequence, it can be deduced that their assay also covered all variants equally (Rabien *et al.,* 2010).

5.2.6   *BMP-6, FGF-8b, MSMB, TRPM8 (II)*

MSMB has been claimed to be in the top three of highly expressed genes in prostate cells (Lilja & Abrahamsson 1988), with lower mRNA levels in malignant tissue than in normal cells (Vanaja *et al*., 2003). It was one the most highly expressed target genes of this study, too (Figure 6), but its mRNA levels in the histologically benign areas and cancerous areas of prostate were here found to be within the same range, with no statistically significant differences between the groups. No association has been found for *MSMB* mRNA levels and clinical stage or Gleason grade (Tsurusaki *et al.,* 1998; Sakai *et al.,* 1999), which is mirrored in the results obtained in this doctoral work. Similarly to *MSMB*, no statistically significant over- or underexpression was seen in the cancerous tissues for *BMP-6, FGF-8b* or *TRPM8* mRNAs, which is in contrast to earlier reports. In the few reports on *BMP-6* mRNA levels in prostate tissue, the gene was claimed to be overexpressed in cancer (Harris *et al.,* 1994; Barnes *et al.,* 1995) and particularly in metastatic cases (Hamdy *et al.,* 1997). Unfortunately in this doctoral

work there were not any specimens available for metastasis-specific analyses. Earlier reports on the overexpression of *FGF-8b* in cancer are contradictory, and some have also claimed an association with Gleason grade, while others did not find any (Dorkin *et al.*, 1999, Wang *et al.*, 1999). *FGF-8b* levels were among the lowest of the studied genes here, supporting the previous reports on low levels of *FGF-8* gene products in cells (Ghosh *et al.*, 1996). *TRPM8* mRNA levels have also been reported to be overexpressed in cancer but with a subsequent decrease in advanced stages (Henshall *et al.*, 2003; Schmidt *et al.*, 2006b). However, these results were not reproduced in this study.

The modest discriminatory power seen here suggests that *BMP-6*, *FGF-8b*, *MSMB* and *TRPM8* are of low importance for the stratification of cancerous and noncancerous prostate tissues. However, for the most abundant prostate-specific genes, the applicability in cancer diagnostics could reside in their use as markers of prostatic origin in the detection of cells in urine or blood, which calls for highly expressed genes to ensure maximum sensitivity. In fact, both *TRPM8* and *MSMB* have been shown to be useful for the detection of prostate cells in both blood and urine of men with metastasized prostate cancer (Bai *et al.,* 2010; Jamaspishvili *et al.,* 2011), which is supported by the fact that their expression levels were among the highest observed in this study as well (Figure 6).

6 CONCLUSIONS

Currently the prominent opinion is that a single biomarker will not be able to solve the problems in prostate cancer diagnostics alone. Instead, a multiplex approach combining the measurements of several target molecules is believed to be a more fruitful focus of research efforts. The aim of this doctoral work was to evaluate the usefulness of 12 candidate genes for prostate cancer diagnostics.

The main conclusions based on the original publications are:

I   *PCA3* mRNA could only be detected in the blood of men with metastasized prostate cancer, but not in healthy controls, or men with localized prostate cancer. Similarly, quantifiable levels of *KLK3* mRNA were only seen in metastatic cases. The qRT-PCR assay developed in this work for detecting *PCA3* mRNA provides absolute mRNA levels without reference gene determination.

II   Cancerous prostate tissue overexpressed *PCA3* mRNA and underexpressed *PSCA* mRNA when it was compared to histologically benign regions in prostates. The observed 5.8-fold overexpression of *PCA3* was not as notable as previously reported. Higher *KLK15* mRNA levels in prostate tissue were associated with advanced pathological stage of the cancer. The expression of the other seven candidate biomarkers, *BMP-6*, *FGF-8b*, *KLK*s 2, 3, 4, *MSMB*, or *TRPM8*, was not significantly different in cancer tissues and, therefore, these genes do not provide discrimination between cancerous and noncancerous samples. However, the expression levels of *MSMB*, *KLK2*, *KLK3*, and *KLK4* were high, and the mRNA transcripts of these genes could potentially be used for a cancer-related detection of prostatic cells in other body fluids such as urine or blood.

III   The mRNAs of prostate cancer-specific fusion gene *TMPRSS2-ERG* were detected in 72% of the prostate cancer cases, and more frequently in cancerous tissue than in benign samples. However, the two mRNA isoforms of the fusion gene were found not only in carcinoma areas, but also in histologically benign prostate tissue of men with prostate cancer, while not in prostates of men without clinical prostate cancer. This could be utilized in assessing the risk of undetected prostatic malignancies even on the basis of histologically negative prostate biopsies. *SPINK1* mRNA was overexpressed in prostate cancer and its lowest tissue levels were found in men without clinical prostate cancer. For both *PCA3* and *SPINK1*, the mRNA levels were higher in histologically benign tissue if the tissue was obtained from a cancerous prostate.

IV Altered expression of *TMPRSS2-ERG* and *PCA3* can sometimes be found in cancerous prostates also in regions that are outside the carcinoma foci. The qRT-PCR assay for *TMPRSS2-ERG* fusion gene mRNAs found more positive areas than the immunohistochemistry experiments for the corresponding ERG protein, and it can therefore be suggested to be a more sensitive method for detecting the fusion. The sample size of the study was limited to cross-sections of five prostates, so further validation is needed for these observations.

Combining the determination of several different nucleic acid levels into a single-reaction RT-PCR test would require careful design and the optimization of the oligonucleotides, other reagents, and reaction conditions to ensure the specific and efficient quantification of all targets. A perhaps more feasible alternative for a multiplex approach would be to measure the analyte levels separately, and subsequently use statistical models to combine the data into an informative measure. The road from finding a potential combination of biomarkers to launching a commercial test for clinical use is long, and requires validation with multiple test panels at several distinct laboratories, and resources for the funding and workforce for this kind of clinical validation were beyond the scope of this doctoral work. Instead, basic groundwork was laid here, and the most promising biomarkers out of the 12 studied candidates were identified. Of the 12, *PCA3* and *TMPRSS2-ERG* were the markers with the best discriminatory power for diagnostic purposes, but potential for prognostic use of any of the studied genes is difficult to assess due to the lack of adequate follow-up times for the studied patients. *KLK15* mRNA levels did however show association with advanced stage. Elsewhere a *PCA3* RNA-based urine test has already been developed, and this PROGENSA® PCA3 test is being offered to clinicians (Groskopf *et al*., 2006), but evidence on its merits on truly improving health outcomes is still insufficient (Bradley *et al*., 2013). There are also efforts currently underway to combine the data on *TMPRSS2-ERG* status with the *PCA3* measurements for improved detection of prostate cancer. In addition to these urine tests, other new, noninvasive tests showing improvements in the field of prostate cancer detection include the 4Kscore™ Test (Vickers *et al*., 2008; Vickers *et al*., 2010) and the Prostate Health Index (phi) (Jansen *et al*., 2010), both combining the protein level measurements of several different prostate-specific kallikreins in serum. Novel marker classes include noncoding RNAs – both long noncoding RNAs (lncRNAs) and shorter RNAs such as microRNAs – but these markers are still at the research stage.

The data obtained in this doctoral work predominantly support the previous findings in medical literature in regard to the biomarkers' cancer specificity and stratification potential and, importantly, this work provides further details on the absolute mRNA levels of the target genes. It is rare in previous works as most of the reported gene expression level data are relative and achieved by normalization to reference genes.

The molecular-level alterations regarding *TMPRSS2-ERG*, *PCA3,* and *SPINK1* mRNAs seen in the histologically benign tissue of men with prostate cancer, but not found in the prostates of men without clinical prostate cancer, can be postulated to be caused by the cancer field effect. This phenomenon and the molecular nucleic acid tests developed in this doctoral work could be utilized for revealing the potentially increased risk of prostate cancer in men who have received negative histological biopsy results. If aberrant molecular levels were detected in their histologically benign tissue biopsies, it could be an indication of a current undetected malignancy in the prostate, or a suggestion of being predisposed to one in the future. In this doctoral work the samples were obtained from prostatectomy specimens, and applying the assays to prostate biopsies could be an interesting future step for validating this hypothesis. In fact, there are already both technical and clinical reports on successful mRNA content determinations in biopsy material (Fukabori *et al.,* 2006; Schneider *et al.,* 2008; Zhao *et al.,* 2011). In addition to assessing the presence of carcinoma, the molecular assays could potentially define a cancer to be of a specific molecular subtype, and this could lead to therapies that would be most beneficial and relevant in the care of a cancer patient.

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