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Individualizace léčby pacientů s karcinomem prostaty na základě molekulární a imunocytochemické detekce cirkulujících nádorových buněk.

Individualization of the treatment of prostate cancer patients based on the immunocytochemical detection of circulating tumor cells

Doctoral thesis

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STATUTORY DECLARATION

I hereby declare that I wrote this dissertation myself and that I have properly cited all sources and literature that I used. I also declare that my dissertation has not been used to obtain any other academic degree.
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IDENTIFICATION RECORD

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ABSTRAKT:

Úvod: Se zavedením nových terapeutických možností u kastračně rezistentního karcinomu prostaty (CRPC) vyvstala i potřeba individuální charakterizace onemocnění pro správnou volbu léčby. Jelikož je běžná biopsie u většiny těchto pacientů nevhodná, může být nahrazena tzv. „liquid biopsy“, tedy analýzou cirkulujících nádorových buněk (CTC) z krve pacienta.

Metody: Metoda AdnaTest (Qiagen, Německo) využívající imunomagnetické obohacení CTC a následnou PCR analýzu vzorků pro přítomnost tumor-asociovaných genů byla testována a použita u 41 pacientů trpících CRPC. Měření bylo provedeno při stanovení diagnózy CRPC a po třetím cyklu terapie docetaxelem. Byl vytvořen a validován panel 27 genů související s volbou terapie u pacientů s CRPC. Genová exprese byla měřena metodou kvantitativní PCR (qPCR) na přístroji BioMark (Fluidigm, USA) a porovnána mezi CTC obohacenými vzorky a bioptickými vzorky primárního nádoru.

Výsledky: CTC byly nalezeny u 85% pacientů v době diagnózy a u 45% pacientů v průběhu terapie docetaxelem. Přítomnost CTC a některých tumor-asociovaných genů, tj. EGFR a AR, souvisela s horší odpovědí na léčbu kvantifikovanou pomocí hladiny sérového PSA (sPSA) a sníženým přežitím. Genová exprese mezi vzorky primárního nádoru a CTC obohacenými vzorky se významně lišila. Semikvantitativní detekce PCR fragmentů metodou AdnaTest korelovala s expresí genů zjištěnou pomocí BioMark. Interindividuální rozdíly v genové expresi byli větší než intraindividuální rozdíly v čase. Sestřihová varianta 7 androgenního receptoru (AR-V7) byla nalezena u 38% AR pozitivních vzorků. Přítomnost AR i AR-V7 souvisela s nižším poklesem sérového PSA. Dvanáct z 27 monitorovaných genů bylo nalezeno i v CTC negativních vzorcích.

Závěr: AdnaTest se prokázal jako metoda vhodná pro detekci CTC v klinické praxi s možností následné charakterizace genové exprese u jednotlivých pacientů. Exprese navrženého panelu genů se liší jak mezi primárním nádorem a CTC obohacenými vzorky, tak mezi vzorky před a v průběhu terapie. Při molekulárně-biologické analýze CTC obohacených vzorků, je třeba brát v potaz přítomnost leukocytární mRNA. Vliv na prognózu a odpověď na terapii byla prokázána u exprese genů asociovaných s AR.

Klíčová slova: cirkulující nádorové buňky; kastračně rezistentní karcinom prostaty; immunomagnetická detekce; personalizace terapie

ABSTRACT:

Introduction: Together with the introduction of new therapeutic options in castration-resistant prostate cancer (CRPC), an advance in individual disease characterization is required. Since common biopsy methods are not suitable for the majority of CRPC patients, one possible solution is the liquid biopsy that is, the analysis of circulating tumor cells (CTCs) isolated from the cancer patients' blood.

Methods: A method based on the immunomagnetic enrichment of CTCs and subsequent PCR detection of tumor-associated genes (AdnaTest, Qiagen) was characterized and used for the detection of CTCs in 41 CRPC patients. Each patient was screened at the time of CRPC diagnosis and after the 3rd cycle of docetaxel therapy. A panel of genes associated with therapeutic decision-making was established and validated. Quantitative PCR (qPCR) method on a BioMark platform (Fluidigm, USA) was used to determine the expression of the gene panel in the CTC-enriched and primary tumor samples and the results were analyzed.

Results: CTCs were found in 85% and 45% of CRPC patients before and during the therapy, respectively. The presence of CTCs, as well as EGFR and AR PCR fragments, was associated with a decreased sPSA response and lower survival. The gene expression of the CTC-enriched and primary tumor samples differed significantly. The semi-quantitative AdnaTest results correlated with the gene expression measured by the BioMark. The Inter-individual differences in gene expression were higher than intra-individual differences at various time points. AR splice variant 7 (AR-V7) was present in 38% of AR positive samples. Both variants were associated with a decreased sPSA response. Twelve out of 27 genes from the monitored panel were found in the CTC negative samples.

Conclusions: AdnaTest proved its value as a CTC detection method in clinical practice and as a liquid biopsy method for individual characterization. The expression of the established gene panel differs between CTC-enriched and primary tumor samples as well as between samples taken before and during the therapy. The presence of mRNA from leukocytes has to be taken into account while using CTC-enriched samples for gene expression analysis. The expression of AR-related genes proved to have a prognostic value and is connected with the therapy response in CRPC.

Key words: circulating tumor cells; castration-resistant prostate cancer; immunomagnetic detection; personalized therapy

TABLE OF CONTENTS:

ABBREVIATIONS:

ACT	beta actin
ADT	androgen deprivation therapy
AKR1C3	aldo-keto reductase family 1, member C3
ALK	anaplastic lymphoma kinase
ALP	alkaline phosphatase
AMACR	alpha-methylacyl-CoA racemase
AR	androgen receptor also AR FN
AR FN	androgen receptor (full length) also AR
AR-V7	splice variant 7 of androgen receptor
BC	breast cancer
BSG	basigin
CD44	cluster of differentiation 44
CD45	cluster of differentiation 45 also leukocyte common antigen
CD56	Cluster of differentiation 56 also neural cell adhesion molecule
cDNA	complementary DNA
CEA	carcinoembryonic antigen
CK	cytokeratin
CLU	clusterin
Cq	threshold cycle number
CRPC	castration-resistant prostate cancer
CSS	cause specific survival
CT	computer tomography
CTCs	circulating tumor cells
CXCL8	chemokine (C-X-C motif) ligand 8, Interleukin 8
CYP17	17 α-hydroxylase/C17,20-lyase
DAPI	4',6-diamidino-2-phenylindole
DHT	dihydrotestosterone
DNA	deoxyribonucleic acid
DSS	disease specific survival
DTC	disseminated tumor cells
Dtx	docetaxel
EAU	European Association of Urology
EDTA	ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor
EMT	epithelial-mesenchymal transition
EpCAM	epithelial cell adhesion molecule
ERBB2	erb-b2 receptor tyrosine kinase 2 also HER2
ERG	erythroblast transformation-specific-related gene
FAM	fluorescein
FDA	Food and Drug Administration
FFPE	formalin-fixed paraffin-embedded
FISH	fluorescence *in situ* hybridization
FITC	fluorescein isothiocyanate
FN1	fibronectin 1
FOLH1	folate hydrolase also prostate-specific membrane antigen 1 (PSMA)
FOXP3	forkhead box P3 anslo scurfin
gDNA	genomic DNA
GS	Gleason score

HER2	human epidermal growth factor receptor 2, also ERBB2
HPRT1	hypoxanthine phosphoribosyltransferase 1
HSD3B2	hydroxy-delta-5-steroid dehydrogenase
IGF1	insulin-like growth factor 1
IGF1R	insulin-like growth factor 1 receptor
IGF2	insulin-like growth factor 2
IL6	interleukin 6
Ki67	marker of proliferation Ki-67
KLK3	kallikrein-3 also PSA
KRAS	kirsten rat sarcoma viral oncogene homolog
LDH	lactate dehydrogenase
LGALS1	lectin, galactoside-binding, soluble 1 also galectin 1
LHRH	luteinising-hormone-releasing hormone
LNCaP	Lymph Node Carcinoma of the Prostate
MMP2	matrix metalloproteinase 2
MMP9	matrix metalloproteinase 9
mRNA	messenger ribonucleic acid
MT3	metallothionein 3
MYC	myc avian myelocytomatosis viral oncogene homolog
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
OS	overall survival
PBS	phosphate buffer solution
PC	prostate cancer
PCR	polymerase chain reaction
PDL1	programmed death-ligand 1
PET-CT	positron emission tomography–computed tomography
PFS	progression free survival
PI3K/AKT	phosphatidylinositide 3-kinases/ Protein kinase B
PMEPA1	prostate transmembrane protein, androgen induced 1
PSA	prostate specific antigen also KLK3
PSMA	prostate specific membrane antigen also FOLH1
PTEN	phosphatase and tensin homolog
qPCR	quantitative PCR
RANKL	receptor activator of nuclear factor kappa-B ligand
RECIST	Response Evaluation Criteria in Solid Tumours
ROS1	receptor tyrosine kinase
RP	radical prostatectomy
RT	radiation therapy
sPSA	serum PSA
SRD5A1	steroid-5-alpha-reductase, alpha polypeptide 1
SRE	skeletal related event
TACSTD2	tumor-associated calcium signal transducer 2
TGFB3	transforming growth factor beta-3
TRAP1	tumor necrosis factor receptor-associated protein 1
TUBB	beta 2A tubulin
UCB	ubiquitin C
VEGF	vascular endothelial growth factor

1 Introduction

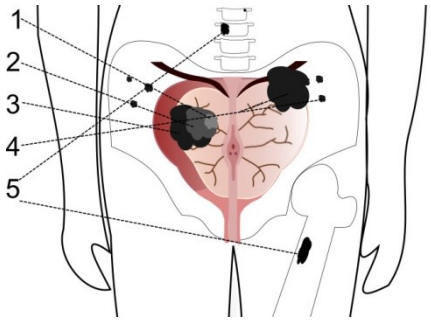
1.1 Prostate cancer

1.1.1 Incidence and mortality

Prostate cancer (PC) is the second most common cancer and the fifth leading cause of death from cancer in the male population worldwide ("Cancer Incidence and Mortality Worldwide", 2013). In Europe, PC is the most common cancer and the third major cause of death caused by cancer in men (Ferlay et al., 2013). The number of PC diagnosis increased rapidly at the end of the 20th century in countries where prostate-specific antigen (PSA) testing for PC detection had been introduced (Potosky et al., 1995). In the Czech Republic, the incidence of PC increased from 60 to 130 patients per 100 000 from 2000 to 2013. Nevertheless, mortality caused by PC did not change very much. In the Czech Republic, PC mortality remained at 30 deaths per 100 000 ("Epidemiologie zhoubných nádorů v České republice", 2005). Such observations were echoed in other countries and led to the question of whether PSA screening brought any improvement to PC treatment. Several large randomized trials designed to resolve this question took place in the USA and Europe, showing conflicting results (Arnold et al., 2015; Haas et al., 2008). Overdiagnosis, leading to unnecessary treatment and high expenditure, is the main drawback of PSA screening (Andriole et al., 2009). PC often progresses slowly, generally affecting older men; thus a fair proportion of patients diagnosed by PSA screening, could live out the rest of their natural lives suffer without suffering from any PC symptoms (Haas et al., 2008). On the other hand, the early detection of PC presents the patient with a very good prognosis (Schröder et al., 2012). According to the last complex review, regular PSA level determination has no effect on overall survival (OS) and presents some benefit only for patients with a life expectancy higher than 10 years (Ilic et al., 2013).

1.1.2 Stages of prostate cancer

PC is classified into several stages and risk groups according to PSA serum level, Gleason score and TNM classification (see Figure 1) (Epstein et al., 2005; “TNM Grading,” 2017).



Risk group	Disease stage	Criteria	Number on the picture
Low-risk	**Localized**	PSA < 10 ng/ml and GS < 7 and cT1-T2aN0M0	1
Intermediate-risk		PSA 10-20 ng/ml or GS 7 or cT2b-T2cN0M0	2
High-risk		PSA > 20 or GS 8-10 or cT3aN0M0	2
Very high-risk	**Locally advanced**	any PSA any GS cT3b-T4N0M0 or anyTN1M0	3, 4
	Metastatic	any PSA any GS anyTanyNM1	5
	Castration-resistant	testosterone < 50 ng/dl and consecutive PSA rise or a radiological progression	not depicted

Figure 1: PC stages and risk groups according to the European Association of Urology (Mottet, 2016).

Almost all PCs are adenocarcinomas developed from the gland cells which produce prostate fluid. Patients with a tumor localized in the prostate gland alone generally have a better prognosis. Patients with very low PSA serum level and favorable biopsy results are evaluated as low-risk patients. Patients with a higher PSA serum level are in the intermediate-risk group and patients with a high PSA serum level and unfavorable biopsy constitute the high-risk group of PC patients. Altogether, the majority of PC patients are diagnosed with the localized disease and their 5-year cancer-specific survival (CSS) is virtually 100%. When the tumor is no longer restricted to the prostate and invades the bladder or seminal vesicles but no metastases are present, disease prognosis is a little worse. Nevertheless, the 5-year CSS of such patients is between 90% and 99%. If metastases appear in the PC they usually affect bones e.g. vertebrae, femur. Bone metastases are connected with pain, the risk of fractures and

a significantly decreased quality of life. Nevertheless, even metastatic PC can be successfully treated by hormonal therapy (e.g. castration). However, the 5-year CSS for metastatic patients is only 30% (American Cancer Society, 2016). The majority of deaths caused by PC occur in patients who progress despite the castration. This stage of the disease is called castration-resistant prostate cancer (CRPC). It is defined by the progression of PSA with or without radiological progression of metastatic lesions with serum testosterone at a castration level. The life expectancy of CRPC patients is presently 12-36 months, depending on metastatic load and the presence of symptoms. Currently, there are no known markers which can predict CRPC development (Hirst et al., 2012; Kirby et al., 2011).

1.1.3 Prostate cancer disease management

Currently, approximately half the patients diagnosed with PC have the low-risk disease. Increasing evidence is being published about the lack of benefits arising from the immediate treatment (e.g. radical prostatectomy), of these patients. It has been shown that depending on the patient's age and comorbidities, active surveillance, or watchful waiting, may increase the quality of life of many patients without influencing their OS. Patients under watchful waiting are treated only according to their symptoms. Patients under active surveillance are regularly tested and start immediate therapy in the case of disease progression. Significant cost and side-effects can be prevented by the application of these approaches (Godtman et al., 2013; Hayes et al., 2013; Mottet, 2016; Stattin et al., 2010).

Complete removal of the prostate, prostatic capsule and seminal vesicles, i.e. radical prostatectomy (RP), remains the main treatment option for patients with the higher-risk localised disease. Although RP may cause side effects, e.g. urinary incontinence and erectile dysfunction, its positive impact on both CSS and OS has been proven (Bill-Axelson et al., 2014). After the operation, the PSA serum level is measured regularly. Since PSA is only produced in prostate cells, its post-RP level is supposed to be below the limit of detection. A repeatedly confirmed increase in PSA serum level above 0.2 ng/ml is evaluated as a sign of disease recurrence (Carthon et al., 2013). For patients suffering from the higher-risk localized disease, radiation therapy (RT) offers a treatment option that is more or less equal to RP in terms of survival (Kalbasi et al., 2015).

Radiation therapy together with hormonal therapy is the standard treatment approach in patients with the locally advanced disease. Since PC is hormone-driven, androgen deprivation therapy is supposed to reduce tumor size, improving the effect of the RT (Bolla et al., 2010). Experience regarding RP followed by adjuvant treatment in locally advanced PC has also

been published. The authors claim an improvement in disease control and patients' OS (Hsu et al., 2007; Mottet, 2016; Ward et al., 2005).

One typical therapy for metastatic PC patients is androgen deprivation therapy (ADT). The deprivation of androgen synthesis can be achieved operatively by a bilateral orchiectomy or pharmacologically by the use of multiple drugs (Pagliarulo et al., 2012). Luteinising-hormone-releasing hormone (LHRH) agonists or antagonists are used for first-line ADT, blocking gonadal androgen synthesis through the inhibition of luteinizing and follicle-stimulating hormone production. Steroidal (e.g. cyproterone acetate) or non-steroidal (e.g. bicalutamide, nilutamide and flutamide) antiandrogens offers another option for ADT. They prevent androgens from interacting with an androgen receptor (AR). An intermittent rather than a continuous ADT is currently under discussion as one possible approach for patients with a long, favorable PSA therapy response. Intermittent ADT may improve patients' quality of life without affecting their OS. Financial considerations also play a role in the introduction of this treatment option (Botrel et al., 2014; Calais da Silva et al., 2009; Verhagen et al., 2014). Current publications provide evidence regarding the improvement of metastatic PC patients' OS when ADT is combined with chemotherapy. Nevertheless, a patient's physical condition has to be taken into account in the decision-making process (James et al., 2016; Sweeney et al., 2015).

Eventually approximately 20% of PC patients develop the castration-resistant (CRPC) form of the disease during the ADT. Not long ago, docetaxel therapy was the only therapy available for these patients. However, new therapeutic options for CRPC patients have been approved in recent years. These are described in the following chapter (Carthon et al., 2013; Mottet, 2016).

1.2 Castration-resistant prostate cancer

CRPC is the final stage of PC with a very bad prognosis and short survival. CRPC is diagnosed when PC progresses despite castration-level testosterone (50 ng/dl). The indication of progression can be biochemical - three consecutive rises in PSA one week apart resulting in two 50% increases over the nadir, and a PSA > 2 ng/ml. Alternatively it may be radiological - the appearance of new lesions, with two or more new bone lesions on a bone scan or the progression of soft tissue lesions using Response Evaluation Criteria in Solid Tumors (RECIST) (Mottet, 2016; Saad and Hotte, 2010).

For many years, this stage of the PC was thought to be no longer dependent on hormones and was called hormone-refractory or hormone-resistant. Nevertheless, it has been proven that AR

is still expressed and often over-expressed in tumor tissue even after a progression to castration-resistant stage. An increase in a PSA serum level whose expression is driven by AR also suggests that AR signaling may be involved in PC progression. Apart from androgen-independent pathways (e.g. p53 mutation and bcl-2 overexpression) suggested for CRPC development, pathways with altered AR signaling have been discovered. The altered AR pathways seem to play a major role in CRPC development and may be induced by androgen deprivation therapy (ADT). On the basis of these discoveries, the term "hormone-refractory" was no longer found to be accurate and the name "castration-resistant" was established as a more appropriate term for this stage of PC (Heidenreich et al., 2011; Scher et al., 2004).

1.2.1 Androgen receptor dependent mechanisms of castration resistance

Much effort has been made in recent years to clarify what enables PC cells to become castration-resistant. Multiple altered AR pathways have been proposed (see Figure 2) with varying degrees of evidence. Most likely, different mechanisms are involved simultaneously in tumor growth which occurs despite androgen deprivation therapy (ADT).

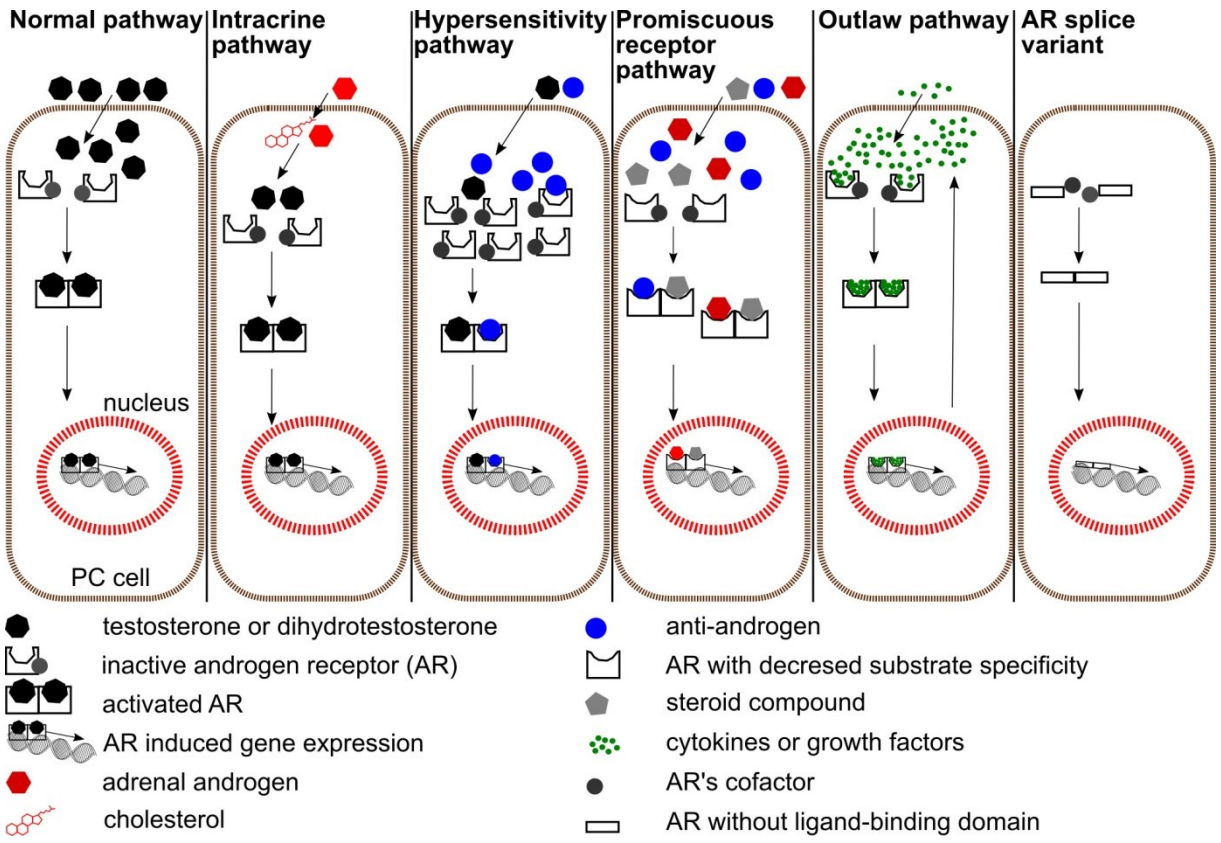


Figure 2: Alternative AR activation pathways in CRPC. Adapted from (Wyatt and Gleave, 2015).

Firstly, tumor growth can be driven by the intracrine pathway. Adrenal androgens, the production of which, in contrast with gonadal androgens, is not affected by conventional ADT, can be transported to cancer cells and there converted to testosterone or dihydrotestosterone (DHT). Both hormones can be also synthesized in cancer cells *de novo* from cholesterol (Montgomery et al., 2008). The intracrine pathway is an innate mechanism of healthy prostate cells which compensates for the low level of testosterone which can endure in some PC cells. It has been shown that enzymes involved in steroid synthesis are over-expressed in the tumor cells of CRPC patients (Stanbrough et al., 2006). The conversion of testosterone to DHT caused by increased 5α-reductase activity was found to be higher in PC cells. The higher activity of 5α-reductase is common in the black population, which is also known to suffer a higher risk of PC diagnosis (Makridakis et al., 1997). Dihydrotestosterone has a several times stronger affinity to AR than testosterone itself, resulting in a longer AR activation even at concentrations much lower than the normal testosterone level. New antiandrogen therapies, such as abiraterone acetate (an inhibitor of the 17,20-lyase activity of CYP17A1), targeting adrenal androgen conversion and testosterone *de novo* synthesis, have

already proven their utility in CRPC patients when combined with the first line of ADT (Mitsiades et al., 2012; Yamaoka et al., 2010).

Secondly, long term ADT can lead to the activation of a hypersensitivity pathway. A constantly low level of testosterone can result in the overexpression of AR or its cofactors by, for example, gene amplification, increased mRNA transcription and mRNA stabilization. It has been demonstrated that approximately 60% of AR overexpression in CRPC is due to X chromosome rearrangement resulting in an increase in AR copy number (Wyatt and Gleave, 2015). The increased expression of AR can lead to the transformation of some AR antagonists, e.g. bicalutamide and flutamide, into weak agonists thus causing treatment failure (Edwards et al., 2003; Chen et al., 2004; Pienta and Bradley, 2006).

Thirdly, AR can be activated either by a ligand other than testosterone or DHT or even by some ligand-independent mechanism. A decrease in substrate specificity can be caused by a mutation in the AR ligand binding domain. This can lead to AR activation by other steroid compounds or even by the antiandrogens used in PC therapy. It was observed that about 40% of patients with ADT would profit from antiandrogen therapy interruption (withdrawal). Some PC patients can have a subpopulation of tumor cells with an AR mutation enabling antiandrogen binding and receptor activation. Their tumor growth thus becomes antiandrogen dependent. After the withdrawal of antiandrogen, tumor growth is delayed until the original signaling is re-established. This mechanism also called the promiscuous receptor pathway, could be an explanation for this antiandrogen withdrawal syndrome. All new antiandrogens, e.g. enzalutamide, are carefully monitored to ensure that they do not cause this syndrome (Hara et al., 2003; Pienta and Bradley, 2006; Small et al., 2004).

Fourthly, the outlaw pathway describes means by which AR can be activated by compounds other than androgens. It has been shown that some growth factors and cytokines, e.g. insulin growth factor, epidermal growth factor, interleukin 6 and interleukin 4, can activate AR when over-expressed (Katsogiannou et al., 2015; Lee et al., 2003a, 2003b; Wu et al., 2006).

Finally, several AR splicing variants completely lacking their ligand-binding domain, such as AR-V7, were identified in CRPC cells. This truncation leads to the continual activation of AR causing PC cell growth and proliferation. It has been shown that these AR splicing variants are connected with a worse prognosis and therapy resistance, even to new antiandrogens (e.g. abiraterone acetate or enzalutamide). For this reason they may have the potential to become prognostic markers and/or new therapy targets (Dehm et al., 2008; Guo et al., 2009; Hu et al., 2009; Nakazawa et al., 2015).

1.2.2 Treatment options in castration-resistant prostate cancer

Until 2010 the only treatment option for CRPC patients with a known OS benefit was docetaxel chemotherapy in combination with prednisone. However, hand in hand with a better understanding of the mechanism of castration resistance, new treatment approaches have appeared. Some new drugs have already proven their efficacy in clinical trials and many others are presently undergoing clinical testing. Docetaxel remains the first-choice treatment for symptomatic CRPC patients but it now can be combined with or substituted by other therapeutic options (see Figure 3) (Tannock et al., 2004).

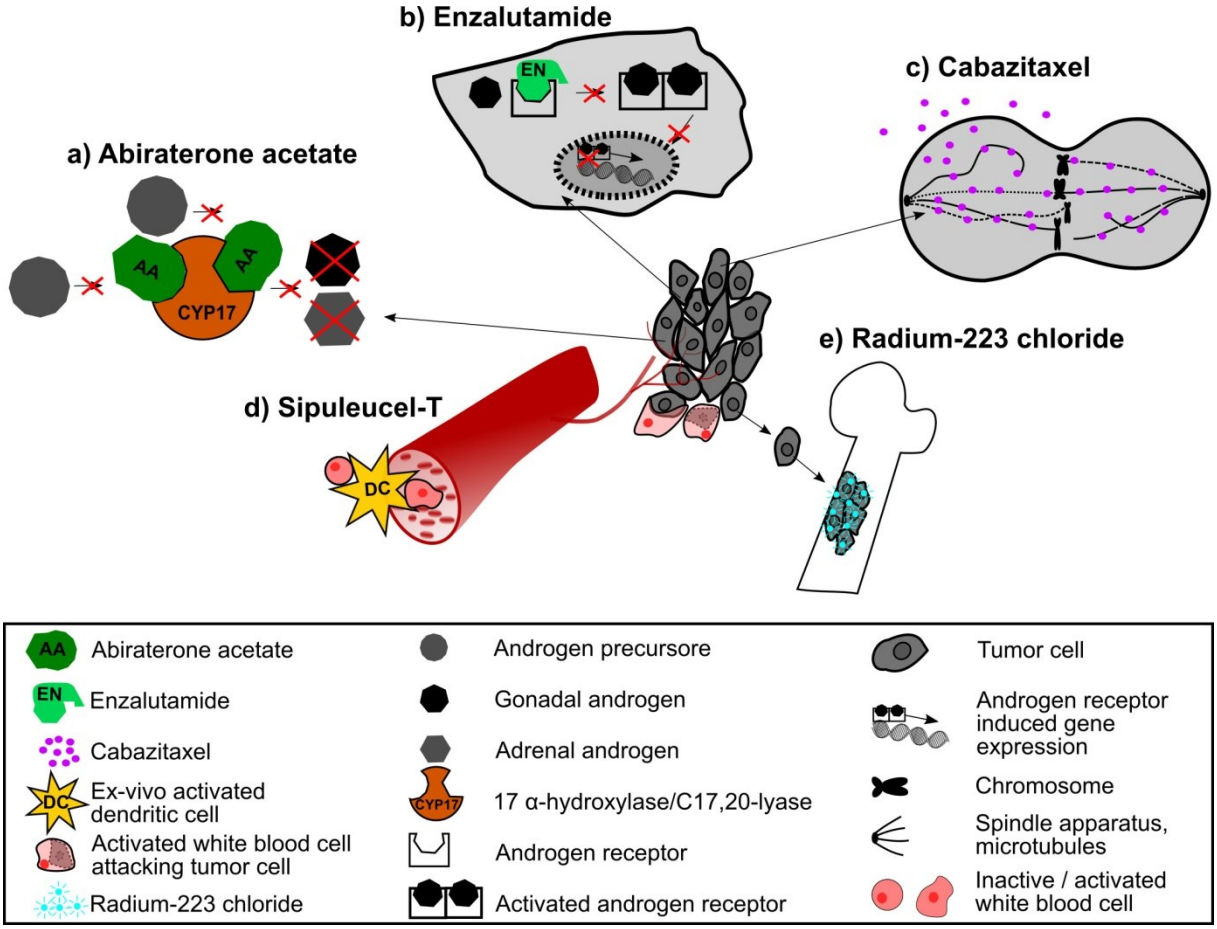


Figure 3: New therapeutics used in CRPC and their mechanisms of action.

a) Abiraterone acetate is a steroidal inhibitor of 17 α-hydroxylase/C17,20-lyase (CYP17), the central enzyme in androgen synthesis. It blocks androgen synthesis in all androgen-producing cells (testicles, prostate, adrenal glands).

b) Enzalutamide is a non-steroidal AR inhibitor. The binding of enzalutamide to AR prevents AR's activation, nuclear translocation and interaction with DNA.

c) Cabazitaxel is a new-generation taxane. It binds to microtubules preventing their normal degradation and synthesis, thus interfering with basic cell functions e.g. cell division.
d) Sipuleucel-T is a vaccine with the patient's own dendritic cells activated *ex-vivo* by a recombinant protein. After their reinsertion in the patient, the cells help to activate white blood cells against tumor cells.
e) Radium-223 chloride is an alpha emitter which is, when introduced, incorporated into bones, because of its similarity with calcium. The majority of radium ends up in the sites with bone metastases where the bone matrix turnover is much higher than in healthy bone tissue: once in place, its radiation kills tumor cells.

Abiraterone acetate, a steroidal selective inhibitor of CYP17 and a new, potent antiandrogen has been approved initially as the second-line therapy for CRPC patients with disease progression despite docetaxel therapy (de Bono et al., 2011). Currently, also stands as the first-line option prior to docetaxel therapy in metastatic CRPC patients both with and without minimal symptomatic disease. Abiraterone acetate has low toxicity and prolongs progression-free survival (PFS) and time until chemotherapy (Ryan et al., 2013). It decreases androgen levels ten times more than common ADT, e.g. LRHR analogues, because it directly blocks the final step of the androgen synthesis, also preventing synthesis from adrenal androgens and cholesterol (de Bono et al., 2011).

Non-steroidal AR inhibitor enzalutamide can now be used, similarly to abiraterone acetate, as both the first and second-line therapy for metastatic CRPC patients (Beer et al., 2014; Scher et al., 2012). Enzalutamide binds to the AR ligand-binding domain competing with androgens, and thus prevents AR nuclear relocation and activation of the androgen response elements of DNA. A potential agonist effect on AR was claimed but just a few antiandrogen withdrawal syndrome observations have been reported suggesting that enzalutamide's agonist effect is rare (Joseph et al., 2013; Korpal et al., 2013; Wyatt and Gleave, 2015). Enzalutamide has relatively low toxicity and unlike abiraterone acetate it is administered alone without corticosteroids such as prednisone. Since corticosteroids cause many side effects and their potential agonist effect on PC cell growth is still unclear, this is a plus for enzalutamide. On the other hand, a current study has proven the good effect of prednisone on OS when combined with docetaxel but not when combined with abiraterone acetate, so the role of corticosteroids in CRPC treatment remains unclear (Teply et al., 2016).

A next-generation taxane, cabazitaxel, represents a second-line chemotherapeutic option in CRPC management. It has shown OS improvement and lower toxicity in comparison with the previously used mitoxantrone in patients in whom docetaxel therapy failed. Cabazitaxel also showed pain palliation which is very important in metastatic CRPC (Bahl et al., 2013; de Bono et al., 2010).

In 2010 sipuleucel-T was approved as a first-line therapy for metastatic CRPC patients. The patient's own leukocytes are activated by an *ex vivo* introduction of a recombinant fusion protein. After the activation, leukocytes are administered in the form of an immunotherapeutic vaccine. After leukapheresis, the vaccine is prepared individually for each patient. Its use is connected with mild side effects such as flu-like syndrome, resulting from infusion administration. Sipuleucel-T has shown OS prolongation but failed in both PSA response rate improvement and PFS prolongation. Other therapies like antiandrogen therapy and docetaxel chemotherapy can follow after the vaccination (Gulley et al., 2016; Kantoff et al., 2010). This therapy is available only in the USA but similar immunotherapeutics for CRPC patients are currently undergoing clinical testing in Europe (Podrazil et al., 2015).

Around 90% of CRPC patients suffer from bone metastases which considerably decrease their quality of life and represent the main cause of death. Not all patients suffering from bone metastases can or want to undergo docetaxel chemotherapy. Radium-223 chloride has been approved as a new treatment option for these patients. An improvement in both OS and quality of life has been shown in CRPC patients with bone metastases treated with radium-223 chloride. Only low myelotoxicity and mild side effects, mostly gastrointestinal, have been observed. Moreover, its non-overlapping mechanism of action makes radium-223 chloride suitable for potential use in combination with other therapies (Parker et al., 2013).

Other bone-protective agents such as bisphosphonates, e.g. zoledronic acid, and receptor activator of nuclear factor kappa-B ligand (RANKL) inhibitors, e.g. denosumab, are also used in the management of CRPC patients with bone metastases. They prolong time to the first skeletal-related event (SRE) thus improving the quality of life but none of them have been shown to prolong OS. However, even after the introduction of new therapies, i.e. abiraterone, enzalutamide, cabazitaxel, sipuleucel-T and radium-223, these drugs are used as supportive and palliative means.

With an increasing number of therapeutic options in CRPC, discussion has begun regarding their optimal sequencing. Based on current evidence, recommendations for CRPC patient subgroups have been proposed (Figure 4). The majority of CRPC patients show the evidence of metastases and are usually further divided according to patients' condition. Such a

condition is graded by performance status from 0 (fully active) to 4 (bedbound). Final treatment decisions can then be taken according to visceral metastases presence, symptoms of disease progression and previous treatment. However, there is also a small group of CRPC patients without evidence of metastases. Little evidence exists about the optimal therapy for these patients. They should be regularly monitored for metastases formation and they are encouraged to participate in clinical trials (Figure 4).

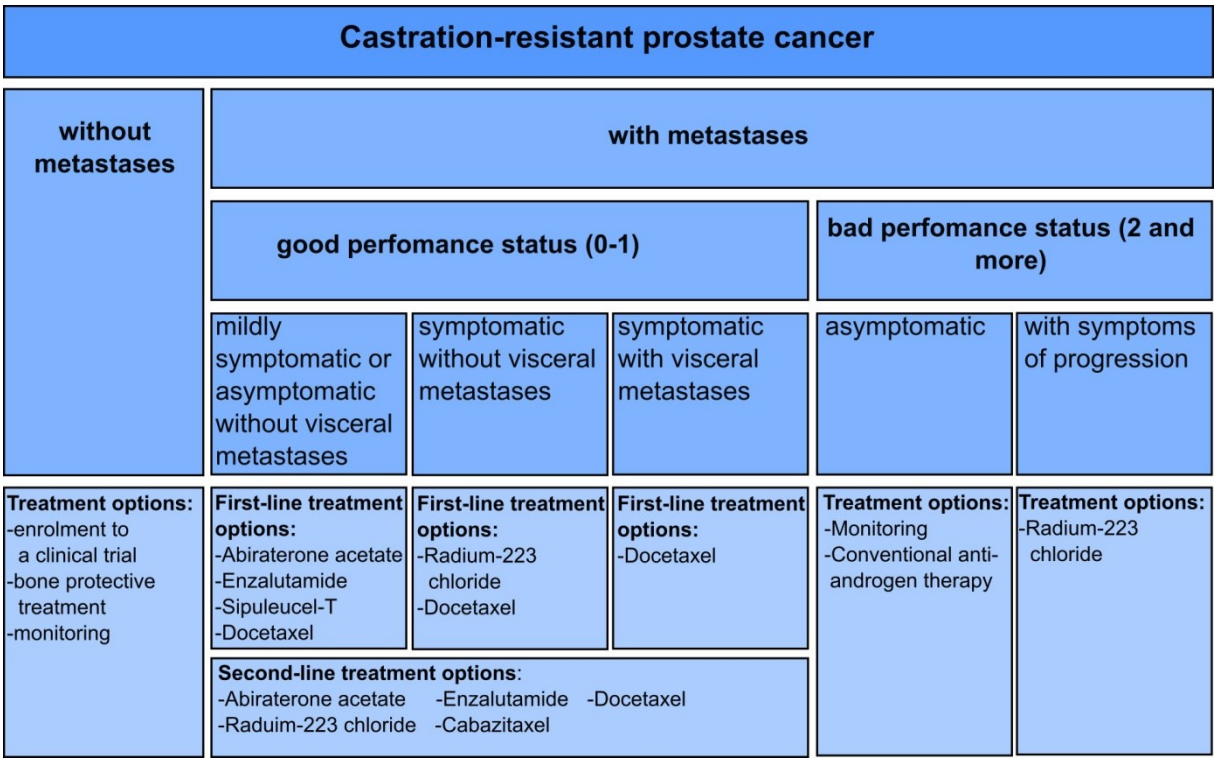


Figure 4: Treatment options and their recommended sequencing in CRPC. Adapted from (Gillessen et al., 2015; Heidenreich et al., 2015).

A question still remains, regarding whether conventional androgen deprivation therapy (ADT) should continue after CRPC diagnosis. Luteinizing hormone releasing hormone analogs, which suppress gonadal androgen production, are often used for the first line of ADT. Until now no prospective study has proven the advantage of such castration continued into a period of disease progression (Taylor et al., 1993). However, the continuing AR signaling in CRPC presumably indicates the positive impact of this approach, especially when combined with the new antiandrogens e.g. abiraterone acetate, enzalutamide.

Similarly, the optimal sequencing of the second and subsequent lines of the therapy remains unclear because of the many possible combinations of the new therapeutics and the short time that has elapsed since their approval. One of the first studies showed that a combination of cabazitaxel followed by abiraterone acetate has a better impact on patient survival than the

converse sequencing of these therapeutics or when just one of them is used (Sonpavde et al., 2015). Similarly, sipuleucel-T showed itself to be the best choice for early CRPC patients with a low disease burden and hormonal agents, such as abiraterone acetate and enzalutamide, yielded good results in the low disease burden patients when administered after the radium-223. Yet the greatest challenge lies in the choice of hormonal agents and taxanes, docetaxel and cabazitaxel, and their sequencing. With the current lack of clinical evidence and a potential cross-resistance between therapies, there is an urgent need for new therapy efficacy and/or resistance markers to help clinicians to make these decisions (Lorente et al., 2015).

1.2.3 Markers in castration-resistant prostate cancer

Alongside the high numbers of ongoing clinical trials and the introduction of new therapeutic approaches, a search for new biomarkers has begun. Surrogate end-point markers are needed for clinical trial evaluation and predictive and therapy efficiency markers are urgently needed in the CRPC disease management decision-making process. Currently, imaging methods such as scintigraphy and PET-CT, and characteristics such as PFS and quality of life improvement are available in decision-making. However, the use of only very few biochemical markers has been proposed (Dancey et al., 2010; Scher et al., 2013).

As well as in other stages of PC, PSA serum level is measured in CRPC. As some of CRPC patients had undergone radical prostatectomy the absolute level of PSA differed strikingly within the group. For this reason, 30% or 50% PSA decline within three months instead of absolute PSA concentration is taken in account in CRPC. According to some clinical studies, PSA decline can serve as a predictive and therapy efficiency marker (Armstrong et al., 2007; Armstrong and Febbo, 2009; Seisen et al., 2016). Nevertheless, some therapies, e.g. immunotherapy, showed improvement in OS without any PSA decline. Even in chemotherapy, PSA flares - PSA serum level growth during first months of therapy before the decline - are known to occur (Kantoff et al., 2010). Since PSA production is driven by AR signaling, PSA decline depends upon the principle of action of a therapy used in specific cases and thus it should be evaluated with caution (Armstrong et al., 2012).

Since the majority of CRPC patients already have or will develop bone metastases, bone turnover markers help in disease status monitoring. Bone matrix resorption and formation products, e.g. N-telopeptide, C-telopeptide, bone sialoprotein and bone alkaline phosphatase, and osteoclast and osteoblast regulation markers, e.g. tartrate-resistant acid phosphatase, osteopontin, osteoprotegerin and RANKL, have been reported as useful markers in CRPC (Coleman et al., 2008; Jung et al., 2004). A higher suppression of urinary N-telopeptide and

serum bone alkaline phosphatase has been connected with the reduction of skeletal related events in patients treated with denosumab (Fizazi et al., 2011). Bone turnover markers are useful in bone-directed therapy efficiency evaluation and bone metastases progression prediction (Armstrong et al., 2012; Armstrong and Febbo, 2009).

Universal cancer-related biochemical characteristics such as lactate dehydrogenase (LDH) and haemoglobin levels also serve as prognostic biomarkers in CRPC. An elevated LDH level - a product of tumor cell metabolism - correlates with disease progression and a worse prognosis. Anaemia has been found to be one of the strongest prognostic markers in CRPC patients. Arising from the disease burden and therapy side-effects, anaemia has been shown to correlate with PSA decline, tumor response rate and OS. LDH and anaemia are both included in nomograms used for CRPC decision-making and clinical trial evaluation (Armstrong et al., 2012; Halabi et al., 2003; Smaletz et al., 2002).

The more new therapies there are, the harder it is for a physician to decide which one would be of greatest benefit to each patient. PSA is a good prognostic and therapy efficacy marker in non-CRPC patients but has a limited use as regards CRPC. Bone turnover markers can supply some information about disease progression but are of no use in non-metastatic diseases or in patients with visceral metastases. The more that is known about the molecular mechanism of CRPC, the clearer it is that future markers will have to be able to identify the specific molecular characteristics of every single patient. Only with a knowledge of each patient's specific mutations and gene splice variants which can lead to the therapy resistance, will it be possible to choose the optimal therapeutic approach. There is an urgent need for predictive and surrogate markers in CRPC disease management and circulating tumor cells (CTCs) appear to be as the best option to fill this gap. Circulating tumor cells can be found in the majority of CRPC patients and they have already proven their utility as a surrogate marker in clinical studies. Moreover, they can be used for the determination of a cancer's molecular characteristics at an individual level (Antonarakis et al., 2014; Bjartell, 2011; Leversha et al., 2009; Mark Thalgott et al., 2015).

1.3 Circulating tumor cells

The first observation of CTCs was made as early as 1869 by Thomas Ashworth during the autopsy on a man who died of cancer (Ashworth, 1869). Later in the 19th century, Stephan Paget published his "seed and soil" theory suggesting a possible mechanism for organ metastasis formation. He pointed out that many types of cancer metastasized preferentially to specific organs and explained this fact in terms of the favorable environment, "fertile soil",

which these organs offered for the specific tumor cells, "seeds" (Paget, 1889). Since this hypothesis has never been fully proven, a lot of supporting evidence has been gathered and the theory of "seeds" encouraged an interest in CTCs (Fokas et al., 2007; Hart and Fidler, 1980; Kinsey, 1960; Sugarbaker, 1952).

CTCs are cells which originate in a tumor or metastasis but have liberated themselves from cell-cell interactions and escaped into the circulation. They are an integral part of cancer dissemination and they provide the physical evidence of an ongoing metastatic process (Fehm et al., 2002). CTCs can be found in the blood of cancer patients and their isolation and characterization provides data on disease progression and individual tumor properties. Thanks to their tumor origin and their possible acquisition from patients' blood, CTCs are often called a "liquid biopsy" (Figure 5).

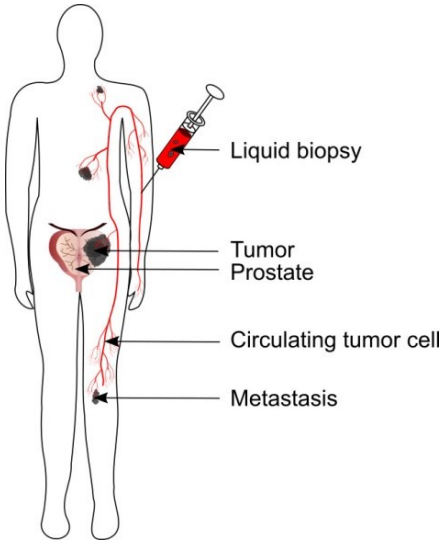


Figure 5: A liquid biopsy, or collection of CTCs from the blood of a cancer patient.

Since this liquid biopsy is actually a common venepuncture it is relatively non-invasive and can be performed repeatedly during therapy. CTC detection thus can serve for the monitoring of even those cancer patients who cannot undergo a standard biopsy. The possibility of cancer monitoring and characterization directly from the patients' blood makes CTCs an exceptionally powerful cancer biomarker. However, until the end of the 20th century the technically challenging extraction of CTCs from the blood, caused by their low numbers in comparison with blood cells, barred their clinical application.

1.3.1 Role of circulating tumor cells in cancer metastasis

A metastatic process begins with the escape of tumor cells from their primary site. This step comprises a complex change in the cells from an epithelial into a mesenchymal phenotype.

This process is called an epithelial-mesenchymal transition (EMT) and it is well-known from embryogenesis. However, EMT also plays a crucial role in metastases formation. EMT enables tumor cells to break their connection with surrounding cells, degrade the extracellular matrix, enter the circulation and survive in the new environment (Yang and Weinberg, 2008). As has been shown on mouse models, CTCs are less adhesive than regular cancer cells, expressing less β4-integrin, E-cadherin and γ-catenin. This and their resistance to pro-apoptotic stress and anoikis, a detachment induced cell death, represent the crucial characteristics needed for their survival in the circulation (Howard et al., 2008). EMT is connected with a down-regulation of epithelial marker expression - such as epithelial cell adhesion molecule (EpCAM) and cytokeratin (CK), and simultaneous expression of mesenchymal markers such as fibronectin, vimentin and slug. CTCs with the EMT phenotype were found in approximately 30% of BC patients and they were related to a more aggressive disease and a worse patient treatment response. Moreover, it was shown that epidermal growth factor receptor (EGFR) is often co-expressed in EMT phenotype cells. A dedicated *in vitro* experiment supported the idea that the EMT switch in CTCs can be activated by the EGFR signaling pathway (Serrano et al., 2014). Yet the CTCs' transition into mesenchymal phenotype does not have to be complete. It has been shown that some CTCs in PC with the invasive phenotype, i.e. able to degrade the extracellular matrix, also express epithelial marker, i.e. EpCAM, leading to the conclusion that full EMT is not essential for the activation of an invasive CTC phenotype (Yao et al., 2014). A single-cell analysis has proven EpCAM expression in the majority of PC CTCs. However, a group of genes (IGF1, IGF2, EGFR, FOXP3, and TGFB3) involved in EMT was also expressed in the greater part of the analyzed cells. Some of the EMT-related genes were expressed more in CTCs from CRPC patients in comparison with castration-sensitive patients supporting the hypothesis about a connection between EMT and a more aggressive disease phenotype (Chen et al., 2013).

Tumor vessels represent another important step in the CTC release cascade. The ability of CTCs to leave their primary site and metastasize is thought to be facilitated by aberrant endothelial cells in the walls of tumor vessels. They facilitate the shedding of tumor cells into the circulation (Chang et al., 2000). Whilst CTCs are detected in the blood, tumor cells enter lymph vessels as well. They can often be detected in the sentinel lymph nodes and bone marrow. Tumor cells found in bone marrow are also termed disseminated tumor cells (DTC) and are thought to represent a pool of dormant tumor cells. They survive in the bone marrow thanks to its fertile environment, with a high concentration of various growth factors present to support the maturation of blood cells. The presence of DTCs in early BC patients has been

connected with a worse prognosis and a late disease flare. The quiescent or dormant fraction of DTCs and CTCs is thought to be the source of the minimal residual disease known especially in BC patients. Yet the connection between DTC and CTC is still unclear. CTCs have been found in some BC patients even many years after primary tumor removal. Based on limited CTC survival in circulation, it is presumed there must be a pool of dividing cancer cells in the patient's body. Thus it is possible that these CTCs may originate from DTCs which have survived in the dormant state in bone marrow (Meng et al., 2004).

The main advantage of CTCs in comparison with DTCs is their detection from the blood which can be performed as often as necessary with minimal discomfort to the patient. In contrast, a bone marrow aspiration needed for DTC detection must be performed by a specialist and it imposes a significant patient burden (Slade and Coombes, 2007).

Even after entering the circulation, the presence of CTCs is not sufficient for metastasis formation. (Hunter, 2004) As already established by Paget, the environment plays a crucial role in the metastatic process (Paget, 1889). This metastatic inefficiency phenomenon was quickly noted in CTC research. It was shown that only about 0.01% of CTCs achieved metastasis formation (Fidler, 1970; Langley and Fidler, 2011). This low efficacy can be due to the fact that a significant proportion of CTCs, as has been shown in advanced BC patients, is apoptotic. Other reasons can lie in a genetic background effect, specific gene mutations or epigenetic changes which have to be present to enable CTCs to form a new metastasis (Méhes et al., 2001). In an *in vivo* test of CTCs' tumorigenic potential only two out of ten mice inoculated by CTCs from other mice with PC developed metastases. None of the mice inoculated by human CTCs showed any symptoms of cancer. Many reasons e.g. sampling, place of sample introduction and a different environment, could contribute to the failure of human CTCs to form a metastasis in mice. It has been proven that CTCs are definitely able to induce cancer metastases, but the process is, as pointed out earlier, surprisingly inefficient (Carvalho et al., 2013). Similarly, a current single-cell analysis of CTCs from CRPC patients confirmed that just a small subpopulation of CTCs isolated from the blood is viable and thus able to form metastases. One or more viable CTCs per ml of blood were found in 22% of CRPC patients. Only about 2% of isolated CTCs survived cultivation for longer than seven days. Just a small subset of isolated CTCs showed some invasive properties, e.g. ability to degrade an intracellular matrix. Together with secretion and expression analysis, the authors deduced that the CTCs which survived cultivation were in a quiescent, non-proliferative state (Yao et al., 2014). This is true at least for the CTCs isolated from blood and cultivated *in vitro*.

To summarize, CTCs are indispensable in cancer dissemination and further CTC research may yield information which provides a key to metastatic process monitoring, individual cancer characterization and treatment personalization. One of the first issues in CTC research is their isolation from the blood. For this reason great effort has been put into the development of CTC isolation and detection techniques.

1.3.2 Isolation and detection of circulating tumor cells

It is assumed that CTCs are released into the blood at an approximate rate of one million cells per day per gram of tumor tissue. Nevertheless, some of these cells are naturally cleared from the blood (Butler and Gullino, 1975). The concentration of CTCs found in cancer patients varies from zero to several thousand cells per milliliter of whole blood (Allard et al., 2004). In comparison with the numbers of erythrocytes (10^9 per ml) and leukocytes (10^6 per ml), seeking CTCs really is akin to looking for a needle in a haystack (Yu et al., 2011). The low number of CTCs in the blood makes their isolation and detection really challenging.

Nevertheless, dozens of techniques for CTC determination have been developed in the last twenty years (Table 1). The methods usually consist of multiple steps. CTCs are isolated or at least enumerated from the sample, detected and their further characterization may follow. Whilst detection and characterization are usually performed by antibody-based fluorescent staining or polymerase chain reaction (PCR) techniques, various approaches have been applied to CTC isolation (Figure 6). **Antibody-based** and **immunomagnetic** methods use the epithelial origin of CTCs for their isolation. Many methods take advantage of the differences between the **physical properties** of CTCs and blood cells. **Functional assays** identify CTCs according to their invasive potential and methods based on **negative selection** remove blood cells on the assumption that the remaining cells will be CTCs (Mikulová et al., 2011; Yu et al., 2011). Currently, many of the new CTC isolation and detection techniques are performed on **microfluidic devices** (Table 1). Some of the devices enable micromanipulation and single-cell analysis. The high purity of captured CTCs unfortunately often comes at the price of slow throughput and high cost (Kirby et al., 2012).

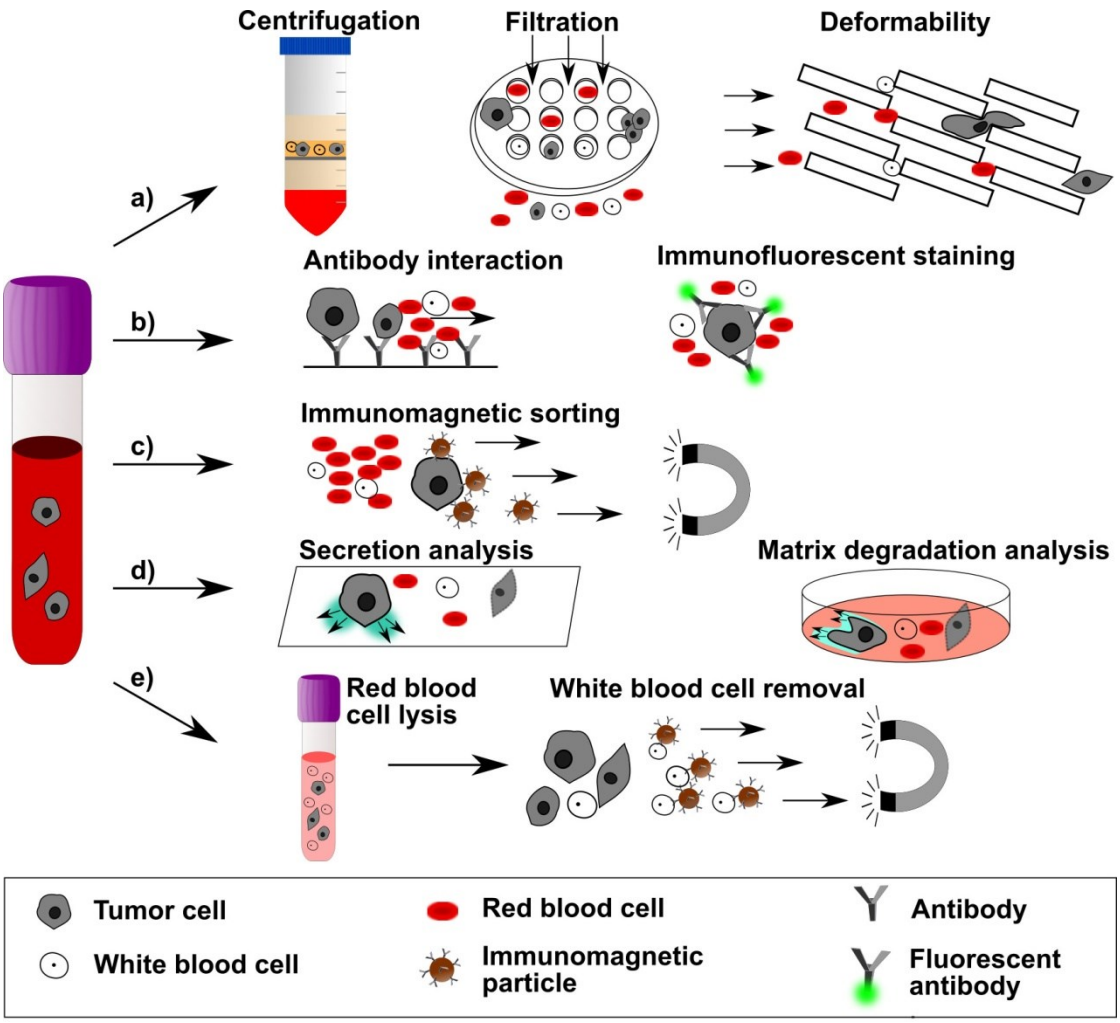


Figure 6: Different approaches to CTC isolation from the blood. a) based on CTC physical properties; b) antibody-based; c) using immunomagnetic separation; d) functional assays; e) negative selection.

Many methods use epithelial antigens present on CTCs to distinguish them from blood cells. Antigens such as EpCAM or CK are often used for **antibody-based** CTC determination. A specific subgroup of these methods uses antibodies attached to magnetic particles for CTC isolation. These methods, termed **immunomagnetic separation methods** e.g. AdnaTest, CellSearch, MACS etc., are currently the most widely used for CTC isolation and detection. Nevertheless, cancer cells are highly heterogeneous and adaptable and, as discussed in the previous chapter, CTCs undergo epithelial-mesenchymal transition (EMT) while in the circulation. This plasticity helps them to survive and to invade new metastatic loci but they may lose their epithelial characteristics during the transformation (Thiery et al., 2009). For this reason, a discussion is underway regarding the use of CTC isolation and detection methods based only on epithelial antigen expression.

The first and only method for CTC detection approved by the U.S. Food and Drug Administration (FDA), CellSearch, is a semiautomatic device using **immunomagnetic selection**. It combines a positive selection of EpCAM+ cells and a negative selection of leukocyte common antigen CD45+ cells (Cohen et al., 2008; de Bono et al., 2009; Daniel F. Hayes et al., 2006). The majority of clinical data known about CTCs were gathered by using CellSearch and the EpCAM positive CTCs, which have proven their clinical validity as a prognostic marker in many clinical studies. Even a current CTC definition, nucleated EpCAM+/CK+/CD45- cell, is derived from this method (Allard et al., 2004). Although many new molecules have been suggested and tested, we still lacka generally-accepted panel of characteristics defining CTCs (Kirby et al., 2012; Nelson, 2010; Satelli et al., 2015). Since the **antibody-based** methods always lead to the selection of just a subpopulation of CTCs, many new methods have been developed with the intention of capturing all CTC subpopulations, not only the EpCAM positive cells.

The most frequently-used approach for CTC isolation is based on the **physical properties** of CTCs (size, deformability, adherence etc.) which differentiate them from blood cells. One of the oldest methods for CTC detection uses density gradient centrifugation to separate larger cells such as mononuclear cells and CTCs from the whole blood. The majority of cancer cells are tend to be larger than blood cells and thus suitable for sorting by filtration. Filtration has become very popular in recent years because of its low cost and high yield. It is very successful in the isolation of CTC clusters. These micro-thrombi, composed of two or more CTCs bound together, have been found in 30-40% of breast cancer, PC and melanoma patients (Cross et al., 2007; Harouaka et al., 2013; Remmerbach et al., 2009; Sarioglu et al., 2015; Sollier et al., 2014). However, the size of the individual CTC from the breast and PC patients was measured as 78-62 μm^2 and 89 μm^2, respectively. This is not so different from size of some leukocytes. On the other hand, it is significantly diminutive when compared with the size of the cells from the common cancer cell lines which were used for the standardization of some filtration-based methods. Filtration thus fails to capture a subpopulation of CTCs, i.e. the CTCs smaller than leukocytes. Moreover, the higher deformability of CTCs may enable even the bigger cells to pass through the filter pores (Cross et al., 2007; Harouaka et al., 2013; Remmerbach et al., 2009; Sollier et al., 2014).

Deformability and adhesion play a part in cell survival, extravasation and tissue invasion and can be distinguishing features between cancer and normal cells. A nanomechanical phenotype of CTCs was shown to be able to distinguish the CTCs of CRPC patients from those of

castration-sensitive PC patients. It could thus serve for the determination and classification of CTCs and for estimating the probability of disease progression (Osmulski et al., 2014).

It is known that not all CTCs isolated from the blood have metastatic potential. **Functional assays** have been developed to identify only those CTCs which are capable of invasion and thus thought to be the most valuable from the clinical point of view. These cells can be identified according to, for example, degrade the extracellular matrix or to secrete cancer-related proteins. In contrast, methods using **negative** cell **selection** remove blood cells with known characteristic from the sample by, for example, erythrocytes lysis or antibody-based leukocyte removal. These techniques prevent the loss of a false negative CTC subpopulation which is a well-known drawback of the other CTC isolation methods. On the other hand, CTCs isolated by negative selection have lower purity which complicates the final evaluation.

Table 1: CTC detection techniques according to their principle of CTC isolation. Methods using microfluidics are indicated by * (Ferreira et al., 2016; Ignatiadis et al., 2015; J. Li et al., 2015).

Physical properties	**Anti-body-based**	**Immunomagnetic separation**	**Negative selection**	**Functional assays**
AccuCyte–CyteFinder	Biofluidica*	AdnaTest	CTC-iChip*	CAM-based Vita-Assay™
Acoustophoresis Chip Size*	CTC-Chip*	CellSearch	EasySep® Human CD45 Depletion Kit	EPISPOT
ApoStream™*	CytoTrack™	CTC-iChip*	EPIC platform	
CellSieve™	FASTcell	Ephesia CTC-chip*	Microfluidic Cell Concentrator (MCC)*	
ClearCell® FX*	GEDI*	IsoFlux*	QMS	
Cluster-Chip*	GEM chip*	LiquidBiopsy*	RosetteSep™	
CyteSealer™	Gilupi	MACS® system		
DEPArray*	Graphene oxide–GO Chip*	Magnetic sifter		
Elasticity-based microfluidic device	HB CTC-Chip*	MagSweeper		
FaCTChecker*	ImageStream	Quadrupole magnetic separator		
Ficoll-Paque®	Modular CTC sinusoidal microsystem	VerIFAST		
Flexible Micro Spring Array (FMSA)	NanoVelcro*			
ISET	OncoCEE™*			
MetaCell®				
OncoQuick				

Parsortix* Parylene filter Photoacoustic flow cytometry ScreenCell Slanted spiral microdevices* Vortex* VyCAP				

Although a great improvement has been made in CTC detection techniques, the best approach for CTC-count evaluation still has to be established. It is assumed that at least one CTC has to be present in every patient with cancer metastasis (Cohen et al., 2008; Coumans et al., 2012b; Cristofanilli et al., 2004; de Bono et al., 2009). Unfortunately, CTC collection and detection strongly depend upon the volume of blood draw. For example, if hundreds of CTCs were present in a patient's circulation the probability of collecting at least one CTC in 7.5 ml of blood would be 50% and the probability of detecting this one cell by one of the most widely used methods, CellSearch, would be circa 18% (Tibbe et al., 2007).

To overcome the problem of low sensitivity, various modifications to current methods have been suggested. An extension of epithelial and cancer-related antigens of stem-cell and EMT markers, e.g. vimentin, has been proposed. These improvements enabled a sensitivity increase of up to 80% (Pal et al., 2015; Satelli et al., 2015). The CTC detection rate can be increased by a combination of methods such as CellSearch plus AdnaTest. The combination of these techniques with different detection methods introduces the possibility of CTC molecular analysis (Giordano et al., 2012; Van der Auwera et al., 2010). So far, the closest to the goal of 100% sensitivity has been the first microfluidic device, the CTC Chip, which detected CTCs in 99 % of metastatic cancer patients (Nagrath et al., 2007). The currently accepted cut-offs of 5 CTCs for prostate and breast cancer and 3 CTCs for colorectal cancer more reflects the inadequacy of the techniques used than any clinical meaning (Tibbe et al., 2007).

The problem with any new method's introduction and validation lies in the lack of a reliable model for CTC isolation and detection. New methods are tested by using tumor cells from cancer cell lines spiked into the blood of healthy donors. However, this method has serious drawbacks. Firstly, it is hard repeatedly and precisely to spike low numbers (units) of tumor cells into the samples. Secondly, CTCs are more heterogeneous in both physical and biological properties than cells from the cancer cell lines (Gleghorn et al., 2010; Powell et al.,

2012). For these reasons, all of the methods' characteristics established in preclinical testing such as capture efficiency and purity are misleading. Many methods have claimed to improve CTC capture and find higher numbers of CTCs in a higher percentage of patients. However, the mechanism of CTC isolation and detection has to be always considered. Since each method uses a different approach, thereby measures different subpopulations of CTCs, the prognostic and predictive abilities of CTCs detected by different methods should be determined individually (Ferreira et al., 2016; Marrinucci et al., 2012; Pecot et al., 2011; Serrano et al., 2014).

A deeper understanding of the roles of various CTC subpopulations in the metastatic process is needed before the final selection of the best CTC detection method for clinical use. However, all CTCs isolated from patients carry important information in their proteins and nucleic acids (Dalum et al., 2012). Especially in the context of various, emerging anti-cancer therapies, an analysis of AR splice variants, EGFR receptor presence and many more molecular characteristics hidden in CTCs could help clinicians the make the therapy really personalized (Ferreira et al., 2016; Kirby et al., 2012).

1.4 Circulating tumor cells in clinical practice

At the end of the20th century, after the development of more sensitive detection methods, CTCs were found in patients with early stage cancer. Since they were not detected in patients with a benign disease, it was thought they could serve as a screening and early cancer detection marker. Unfortunately it was soon clear that CTCs can be found only in a low percentage of early cancer patients (Racila et al., 1998). However, interest in CTCs has been restored. Currently, the majority of CTC research is focused on advanced and metastatic cancer. The high rate of CTC positive patients as well as the higher absolute numbers of CTCs facilitate their detection and have enabled the establishment of CTCs as a cancer biomarker.

1.4.1 Circulating tumor cells as a cancer biomarker

Different approaches have been applied for the use of CTCs as a cancer biomarker. At first, the absolute number of CTCs was established as a prognostic marker of survival in advanced breast, prostate and colorectal cancer. Later, the molecular characterization of CTCs revealed its potential in non-invasive cancer profiling and therapeutic decision-making (Table 2).

Table 2: Examples of the use of CTCs as a marker in various cancer types (Bidard et al., 2016; Huang et al., 2016; Krebs et al., 2010; Zhang et al., 2015)

Type of cancer	**CTC count as the cancer marker**	**CTCs as a liquid biopsy (purpose of determination)**
Metastatic breast cancer	≥ 5 CTCs unfavorable prognostic marker for OS and PFS at any time during therapy Predictive marker of survival	HER2 status (therapy sensitivity) Ki67 (therapy sensitivity) PDL1 (therapy sensitivity)
Metastatic colorectal cancer	≥ 3 CTCs unfavorable prognostic marker for OS and PFS at any time during therapy Predictive marker of survival	EGFR mutations (therapy resistance) CEA mRNA detection (disease monitoring) KRAS, mutation (therapy response)
Metastatic lung cancer	≥ 5 CTCs unfavorable prognostic marker for OS and PFS at any time during therapy Predictive marker of survival	EGFR mutations (therapy resistance) CD 56 expression (non-invasive tumor profiling) ALK rearrangement (non-invasive tumor profiling) ROS1 rearrangement (therapy resistance)
Castration-resistant prostate cancer	≥ 5 CTCs unfavorable prognostic marker for OS and PFS at any time during therapy	EGFR expression (therapy sensitivity) AR amplification (non-invasive tumor profiling) MYC gain (non-invasive tumor profiling) ERG rearrangement (disease prognosis) Chromosome gene copy number (non-invasive tumor profiling) PTEN amplification (non-invasive tumor profiling)

HER2-Human epidermal growth factor receptor 2; Ki67-Marker of proliferation Ki-67; PDL1-Programmed death-ligand 1; EGFR-Epidermal growth factor receptor; CEA-Carcinoembryonic antigen; KRAS-Kirsten rat sarcoma viral oncogene homolog; CD56-Cluster of differentiation 56 i.e. Neural cell adhesion molecule; ALK-Anaplastic lymphoma kinase; ROS1-Proto-oncogene tyrosine-protein kinase ROS; AR-Androgen receptor; MYC-V-myc avian myelocytomatosis viral oncogene homolog; ERG- erythroblast transformation-specific-related gene; PTEN-Phosphatase and tensin homolog

In 2004, Allard et al. provided the first complex overview of CTC presence in cancer patients and their potential use as a cancer biomarker. By measuring more than two thousand samples of metastatic cancer patients, non-malignant patients and healthy donors it was demonstrated that CTCs could distinguish between benign and malignant disease. CTCs were found in 36% of metastatic cancer patients. The highest rates of CTC positive patients identified were suffering from a PC (57%) followed by breast and ovarian cancer (37%), colorectal cancer (30%), lung cancer (20%) and other cancers (26%). CTC counts ranged from zero to 23 618 cells per 7.5 ml of the whole blood. The highest absolute numbers of CTCs were measured in breast, prostate and lung cancer patients. Results evaluation revealed a high heterogeneity of

CTCs in size (from 4 to 30 μm) and morphology e.g. elongated cells, multinucleated cells, cell clusters and apoptotic cells (Allard et al., 2004).

Shortly after Allard, Cristofanilli et al. published their study on CTC changes during therapy e.g. chemotherapy, immunotherapy, hormonal therapy, in metastatic breast cancer patients. An unfavorable CTC count (≥5 CTCs) predicted both shorter PFS (2.7 vs. 7.0 months) and OS (7.1 vs. >18.0 months). Moreover, patients who changed to favorable numbers of CTCs during the therapy had a better survival rate than those who did not. CTCs were evaluated as the strongest predictor of survival in comparison with the number of previous therapies, the therapy type, the Eastern Cooperative Oncology Group (ECOG) score, the time to metastasis and hormonal receptor status (Cristofanilli et al., 2004). Based on these data, the FDA approved the first CTC detection method, CellSearch (Janssen Diagnostics, USA), as a disease-monitoring technique in metastatic breast cancer patients and approvals for prostate and colorectal cancer soon followed (Cohen et al., 2008; de Bono et al., 2008).

In metastatic colorectal cancer, the absolute number of EpCAM positive CTCs was found to be significantly lower in comparison with breast and PC. The percentage of patients with an unfavorable CTC count (≥3 CTCs) was just 26%. In contrast, when measured by another method which enables CTC identification based on multiple different markers (AdnaTest, Qiagen, Germany), e.g. epidermal-growth factor receptor (EGFR) and mucin (MUC), 81% of patients proved to be CTC positive. In conclusion, CTC count proved to be a strong prognostic marker for both progression-free and OS, but EpCAM positive cells were much less frequent in colorectal cancer (Cohen et al., 2008; Raimondi et al., 2014).

The strength of CTCs as a predictor of survival was confirmed during the Cristofanilli study follow-up period. CTC elevation in any time during of therapy was a predictor of disease progression. CTC count was thus suggested as a decision-making marker for a change in therapy (Daniel F Hayes et al., 2006). Subsequent analysis of two large studies proved that a decrease in CTC count indicated therapy efficacy. In line with this, every increase in the CTC count by an order of magnitude led to an average decrease in patient survival expectancy by six and a half months. A period of up to three months was recommended as a waiting period for final therapy evaluation, because an early CTC decrease followed by an increase was observed in some patients. This was explained by the elimination of the therapy-sensitive CTC subpopulation, followed by disease progression caused by the therapy-resistant cells (Coumans et al., 2012a, 2012b; Cristofanilli et al., 2004; Johann S de Bono et al., 2008).

After the establishment of CTCs as a prognostic marker, their use in clinical studies began. The decrease in CTCs in patients was used as an end-point marker in the clinical study of the

target of rapamycin complex 1 inhibitor. Median CTC decline in the study group was 48% and 72% of patients showed at least some decrease in CTC numbers (Armstrong et al., 2013). In a study performed by our laboratory, CTCs in early BC patients were measured. CTCs were found in 40% of patients prior to neoadjuvant therapy and in 25% of patients after therapy, suggesting a strong therapeutic impact on the majority of the patients. In 20% of patients with detectable CTCs the expression of HER2 was detected despite the HER2 negative status of the primary tumor (Mikulová et al., 2014). In the SWOG S0500 clinical trial the increase in CTCs after first-line chemotherapy identified a group of patients resistant to the multiple commonly-used chemotherapeutics, i.e. those who could benefit more from participation in clinical trials (Smerage et al., 2014). According to these results it is clear that CTC analysis can reveal therapeutically important facts in BC patients.

Nowadays, CTCs are commonly used in clinical practice for the monitoring of metastatic breast cancer, lung cancer, colorectal cancer and PC. They serve as the end-point marker in many ongoing clinical trials (Ignatiadis et al., 2015). Next to the absolute CTC count, the importance of CTC molecular analysis is a recurring theme. CTCs as a liquid biopsy can be used to identify molecular markers of targeted therapies and therapy sensitivity/resistance e.g. HER2, PTEN, VEGF, in various cancer types (Table 2). Oestrogen receptor status and HER2 positivity, which can both be determined in CTCs, is a very important therapy target in BC. EGFR mutation, ALK and ROS1 rearrangement, also determined in CTCs, help in therapeutic decision-making in lung cancer patients (Pérez-Callejo et al., 2016). A detailed description of CTCs' specific role in CRPC, e.g. prognosis determination, AR presence and AR splice variants, is given in the following chapter (Nakazawa et al., 2015).

1.4.2 Circulating tumor cells as a marker in castration-resistant prostate cancer

1.4.2.1 Circulating tumor cell count

The high detection rate as well as the high absolute numbers of CTCs in the blood of prostate PC patients encouraged further CTC investigation (Allard et al., 2004). The optimal target for the introduction of the new biomarker is CRPC with its ongoing metastatic process, lack of prognostic and predictive biochemical markers and urgent need for surrogate markers for clinical studies testing new therapeutics. The studies in Table 3 have proven that CTCs are present in the majority of CRPC patients in counts of up to thousands of cells per milliliter of

blood. Although CTCs have also been studied in other stages of PC, their clinical significance in CRPC is of the greatest importance (Thalgott et al., 2013a).

Table 3: CTC counts and detection rates measured in castration CRPC patients.

CTC detection method	**Number of patients**	**Threshold**	**CTC positive patients**	**Median CTC count**	**CTC range (per 7,5 ml of blood)**	**Citation**
CellSearch	123*	≥5 CTCs	41%	13	72+-333	(Allard et al., 2004)
Immunomagnetic separation for EpCAM+cytometry	26	≥5 CTCs	69%	-	0-8586	(Moreno et al., 2005)
CellSearch	63	≥5 CTCs ≥3CTC	65% 79%	-	0-847	(Shaffer et al., 2007)
CellSearch	120	≥5 CTCs ≥3CTC	57% 75%	9	0-1958	(Daniel C Danila et al., 2007)
CellSearch	231	≥5 CTCs	56% (decreased to 39%,33% and 31% during treatment)	-	-	(de Bono et al. 2008)
CellSearch	119	≥5 CTCs ≥50 CTCs	50% 18%	6 (decreased to 3 and1 during treatment)	0-545 (changed to 0-1317, to 0-1144 during therapy)	(Olmos et al., 2009)
CellSearch	100	≥4 CTCs	51%	4	0-2572	(Goodman et al., 2009)
CellSearch	156	≥5 CTCs	54% (decreased to 30% during therapy)	-	-	(Scher et al., 2009)
AdnaTest	16	0,30 ng/µl	69% (decreased to 31% during therapy)	-	-	(Todenhöfer et al., 2012)
CellSearch	40	≥5 CTCs	57%	7,5	0-2347	(Thalgott et al., 2013a)
CellSearch	89	≥5 CTCs	66%	16	0-1014	(Bitting et

			(75% at progression)	(42 at progression)	(0-1051 at progression)	al., 2015)
CellSearch	215	≥5 CTCs	47%	-	-	(Goldkorn et al., 2015)
AdnaTest	37	0,15 ng/µl	87% (decreased to 53% during therapy)	-	-	(Čapoun et al., 2016)

* Not all patients suffered from CRPC, some possibly suffering from a different stage of PC.

The presence of CTCs was shown to be the strongest predictive parameter of OS in CRPC patients in comparison with the PSA serum level and PSA doubling time. The difference in OS between patients with favorable and unfavorable CTC counts was 2.5 years in contrast to 0.5 years, respectively. Higher CTC counts were also observed in metastatic CRPC patients with disease progression. Although the CTC count within one individual patient did not change significantly, chemotherapy caused high fluctuations in it (Moreno et al., 2005, 2001). These preliminary results were confirmed by de Bono et al. on 231 CRPC patients. Patients with a favorable CTC count, both before and during therapy, showed significantly better OS (Figure 7). Moreover, had proved a better survival rate to those whose CTC count worsened (Figure 7). CTCs were a better predictor of OS than a 30% decrease in sPSA and they were suggested as a surrogate biomarker in clinical studies and also as a decision-making marker for inefficient treatment termination (de Bono et al., 2008). On the basis of this study, the determination of CTCs by CellSearch was approved by the FDA to become a prognostic and therapy monitoring marker in CRPC patients (Goodman et al., 2009).

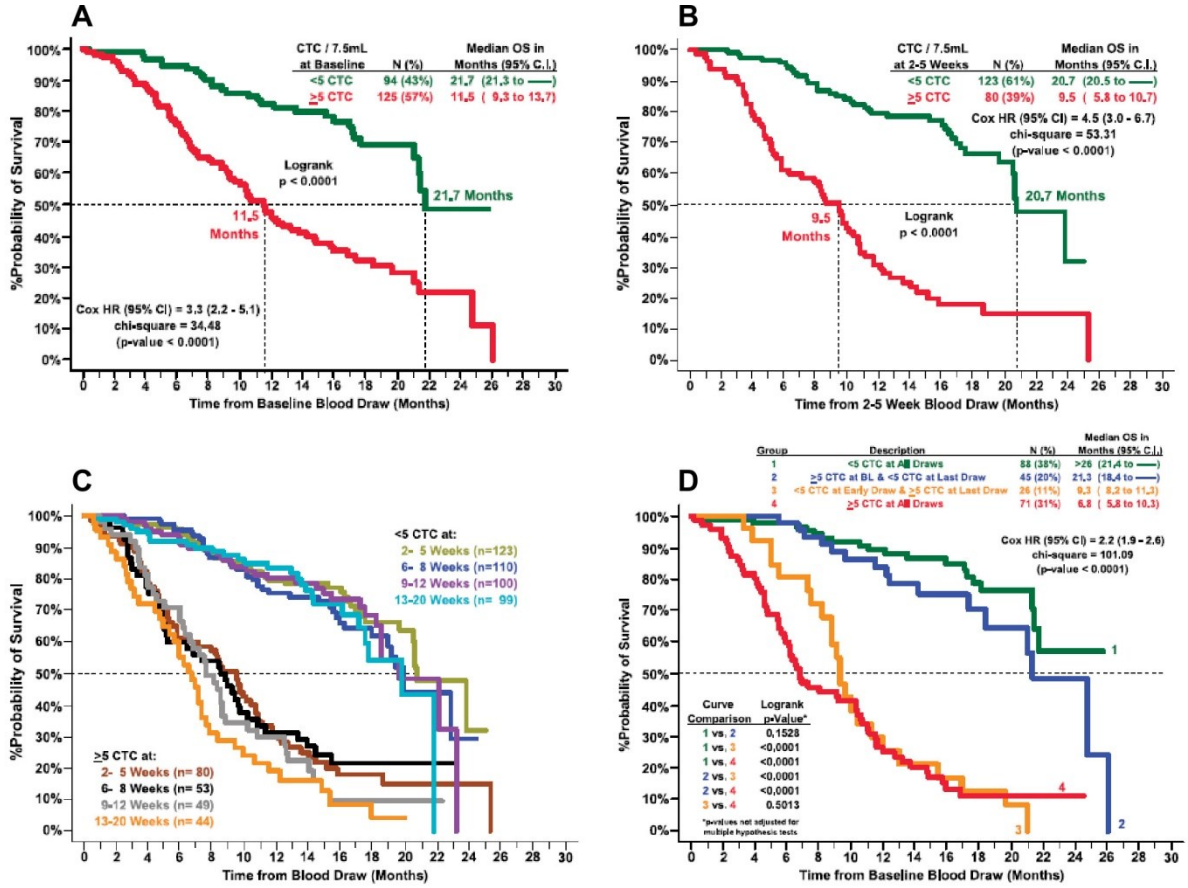


Figure 7: Division of CRPC patients according to favorable (<5) and unfavorable (≥5) CTC count, both before (A) and during (B, C, D) therapy, led to their stratification according to OS (de Bono et al., 2008).

Subsequent studies confirmed the prognostic power of CTCs and nowadays CTCs are a well-established marker of OS in CRPC. The role of CTCs in disease progression and their correlation with biochemical markers has also been widely studied. Higher numbers of CTCs were observed in CRPC patients with bone metastases and those who had previously undergone chemotherapy (Daniel C Danila et al., 2007). Similarly, Thalgott et al. found the lowest CTC counts in patients with only visceral and no bone metastases. The highest percentage of patients (93%) with measurable CTCs was detected in the group of CRPC patients who developed chemotherapy resistance (Thalgott et al., 2013a). The correlation between CTCs and bone metastases was explained by i) their direct contact with bone marrow ii) the higher aggressiveness of cancer cells which cause bone metastases iii) the fact that tumor cells from lymph node metastases are filtered by the lymphatic system (Daniel C. Danila et al., 2007; Goodman et al., 2009; Olmos et al., 2009; Scher et al., 2009). In conclusion, CTCs seem to be able to identify patients endangered by a haematogenous dissemination which cannot be discovered by standard laboratory tests (Bitting et al., 2015).

In contrast with consistent results regarding the relationship between CTCs and bone metastases, contradictory results have been published about their relationship with other biochemical markers. A positive correlation seems to exist between CTCs and the serum level of ALP and LDH. (Bitting et al., 2015; Olmos et al., 2009). However, the association between CTCs and haemoglobin and between PSA serum level and PSA doubling time remains uncertain. The CTC count correlated with PSA serum levels in some studies but showed discordance in others (Moreno et al., 2001; Olmos et al., 2009; Scher et al., 2009). Recently preformed single-cell analysis has shown that only some CTCs secrete PSA. Thus the sPSA in CRPC patients is probably secreted mainly by tumor cells localized in metastases and does not have to correlate with the CTCs (Yao et al., 2014).

Although CTC counts have proven their value as a biomarker in CRPC, the threshold for an unfavorable CTC count has repeatedly been challenged. Cut-offs of three, four or five CTCs were suggested in different studies (de Bono et al., 2008; Goodman et al., 2009; Thalgott et al., 2013a). The accuracy of CTC detection has be viewed with caution when employing as a cut-off such very low CTC counts (Allan and Keeney, 2010). No CTC threshold effect was found in the Danila's study thus the evaluation of CTCs as the continuous variable was suggested (Daniel C Danila et al., 2007). As the continuous variable, CTC count also proved to be associated with shorter survival in the IMMC38 clinical trial. However, the association was weakened by the high range of survival times in patients with low numbers of CTCs (Scher et al., 2009). Latest analysis shows that a cut-off of five CTCs leads to the better prediction of OS than continual CTC counts. To be useful as a continuous variable, CTCs would have to be sampled several times during the three months of the therapy, while their predictive power is strong enough even after the first measurement when cut-off is applied (Mark Thalgott et al., 2015).

Besides the cut-offs, different subpopulations of CTCs were also proposed CRPC marker. Coumans et al. investigated EpCAM+CK+ objects (both with and without a nucleus) found in the blood of CRPC patients. Surprisingly, the number of objects from each subgroup correlated well with the patient OS. Nevertheless, the lowest background was observed in the group of nucleated EpCAM+CK+ cells defined according to criteria commonly used for CTC detection by CellSearch. It was suggested, that the other subpopulations were just life and death stages of these CTCs (Coumans et al., 2010).

A new subpopulation of CTCs, EpCAM+HER2+ cells expressing PSA, PSAM or EGFR, was analyzed in the first study evaluating the AdnaTest method in CRPC. Sixty nine percent and 31% of patients were found CTC positive before and during chemotherapy, respectively. PSA

was expressed in the majority of CTC positive patients. PSMA and EGFR were expressed in 50% and 31% of patients, respectively. The expression of EGFR was connected with radiological progression. This study proved AdnaTest to be a useful method in CRPC monitoring and a source of clinically important data (Todenhöfer et al., 2012).

1.4.2.2 Circulating tumor cells as a liquid biopsy

Besides the prognostic and predictive power of the absolute numbers of CTCs, the molecular profile of CTCs can help with individual disease characterization and differentiate the prognosis of patients with equal CTC counts (Goldkorn et al., 2015). Genetic alterations important for cancer prognosis and therapy sensitivity were detected in CTCs, e.g. AR gene amplification, v-myc avian myelocytomatosis viral oncogene homolog (MYC) gain, phosphatase and tensin homolog (PTEN) loss and erythroblast transformation-specific related gene (ERG) rearrangement. Moreover, proteins connected with therapy resistance and disease invasiveness, e.g. epidermal growth factor receptor (EGFR) expression, telomerase activity, stem and epithelial-mesenchymal transition (EMT) related proteins and AR expression, can also be found in CTCs. Currently, AR splice variant determination in CTCs is revealing its power as a therapeutic decision-making marker.

The clinical utility of cancer-related genetic modifications found in CTCs has been demonstrated in multiple studies. In 2007, AR gene amplification, one of the mechanisms leading to castration-resistance status, was studied by fluorescence *in situ* hybridization (FISH) on CTCs. Amplification was detected in 55% of samples. Although preliminary, this study showed the presence of various CTC subpopulations both within and between patients and proved that CTCs can be used in clinical practice as a liquid biopsy (Shaffer et al., 2007). Later the gene copy number alterations in CTCs were also determined by Scher's group. AR amplification and MYC gain, connected to disease progression and aggressiveness, were identified in 38% and 56 % of CTC samples, respectively. The PTEN loss, a known tumorigenic factor, was detected in 37% of CTCs and correlated strongly between CTCs and tumor biopsy samples (84%). PTEN loss in CTCs was connected with worse OS. More genetic abnormalities were present in the samples with higher CTC counts. Increased chromosomal instability was connected with increased tumor aggressiveness. Interestingly, the presence of AR amplification and MYC gain in CTCs was higher than in solid tumors suggesting that CTCs represent the later generation of cancer cells. In contrast, ERG gene rearrangement, a marker of poor prognosis, was consistent within CTCs from each patient.

ERG rearrangement is thus thought to occur earlier in cancer development while other genetic abnormalities seem to appear later during the disease (Attard et al., 2009; Leversha et al., 2009; Punnoose et al., 2015).

Along with genetic cancer markers, proteins expressed in CTCs can also be used in prognosis determination and therapeutic decision-making. The expression of stem-cell and EMT-related genes in CTCs was related to shorter OS. Interestingly, in the group of patients with favorable CTC counts, a subgroup of patients with worse OS and the expression of stem-cell related genes was identified. Similarly, detection of the activity of enzyme telomerase in CTCs, an unfavorable cancer-related phenomenon, distinguished between patients with longer or shorter survival in the group of patients with intermediate (6-54) CTC counts (Goldkorn et al., 2015; Chang et al., 2015). CTCs with an invasive phenotype which were able to digest collagen matrix were significantly less frequent in castration sensitive than in CRPC patients, suggesting the clinical relevance of this ability (Paris et al., 2009). The expression of EGFR, a therapy target and a potential marker of chemotherapy resistance, was repeatedly detected in circa 40% of CTCs in CRPC patients. The presence of EGFR positive CTCs was connected with a worse prognosis and shorter survival (Okegawa et al., 2016; Shaffer et al., 2007). CTCs can be also used for an *ex-vivo* taxane sensitivity determination. A difference in microtubule bundling was observed in CTCs after docetaxel or paclitaxel treatment, suggesting differing sensitivity of the tested CTCs, which could be used in taxane therapy choice (Kirby et al., 2012).

The specific location within proteins detected in CTCs belongs to AR. The majority of treatment options in CRPC interact with androgen synthesis, AR activation and/or the AR signaling pathway. Since a standard biopsy is a great burden for patients in the late stages of PC, AR status monitoring via CTCs is a perfect option for CRPC patients. If AR is present, AR targeted therapies can be applied; if not, the other therapeutic options can be used without delay.

The heterogeneity of AR expression within CTCs and between CTCs and the primary tumor has been observed in multiple studies. Most CRPC patients exhibited AR-mixed status with AR signaling active in some CTCs and inactive in the others. Patients with this phenotype had worse OS, possibly because of the difficulty of therapy optimization. Moreover, it was suggested that AR status change can be induced by the introduction of androgen deprivation therapy (ADT) introduction (David T Miyamoto et al., 2012). Higher AR expression was found in patients with prior abiraterone treatment probably as a compensation mechanism pertaining to androgen levels. The combining of abiraterone therapy with AR antagonists, e.g.

enzalutamide, could thus prevent this side effect (Punnoose et al., 2015; Reyes et al., 2014). AR localization was also hypothesised as capable of revealing taxane therapy efficacy. The effect of taxanes on microtubules should prevent AR nuclear trafficking. For this reason the nuclear localization of AR may be an indicator of a patient's resistance to taxanes. As expected in advanced patients, the majority (63%) of ARs in CTCs were found in the nucleus. However, the localization of AR was heterogeneous both within and between the patients (Tagawa et al., 2015). All these approaches represent possible applications of CTCs in disease management. However, the most promising application is currently considered to be splice variant 7 of the androgen receptor (AR-V7).

In 2014, Antonarakis et al. described AR-V7, the androgen receptor splice variant without the ligand binding domain, as being expressed in PC. AR-V7 is supposed to be continuously active, thus causing resistance to antiandrogen therapies. AR-V7 was detected in 39% and 19% of CTCs isolated from CRPC patients treated with enzalutamide and abiraterone acetate, respectively. Patients with ARV-7 in CTCs had worse PSA response, progression free and OS (Antonarakis et al., 2014). ARV-7 expression was found to be connected with the EMT phenotype, stem-cell signature and was decreased by DHT presence. While taxane treatment led to the disappearance of the AR-V7 in the CTCs, its expression was enhanced by the ADT. Most probably, AR-targeted therapy put a selective pressure on tumor cells leading to the survival of the tumor cells which were able to avoid its effect by expressing AR-V7 (Kong et al., 2015; Nakazawa et al., 2015). When following the AR-V7 dynamics in a patient's CTCs, taxane treatment can be introduced to those who have developed resistance to AR-directed therapy and it could make render them perceptive again (Nakazawa et al., 2015). However, AR-V7 was found more often in patients treated with abiraterone than those treated with orteronel, a new non-steroidal antiandrogen, and the correlation between AR-V7 positivity and PFS and OS was not observed in this study (Onstenk et al., 2015).

In conclusion, AR and AR-V7 detection in CTCs offers a breakthrough in therapeutic decision-making. The power of AR determination in CTCs lies in the possibility of testing it repeatedly throughout the whole course of therapy and to react to the patient's current AR status by choosing the most efficient therapy. Since AR and its splice variants represent one of the mechanisms of treatment resistance in CRPC the potential for its determination would finally enable the performance of truly personalized medicine (Sprenger et al., 2015).

1.4.2.3 Use of circulating tumor cells in clinical studies

As soon as the potential of CTCs was clearly shown it was just a matter of time before they started to be used in clinical trials. CTCs have become a surrogate marker for patient survival in clinical studies performed on CRPC. Moreover, further analysis of CTCs has often helped to uncover newly tested therapeutics' mechanisms of action and to define target groups of patients (Table 4).

Table 4: Clinical trials and clinical studies comprising CTC detection.

Name of the trial	**Study aim**	**CTC detection method / cut-off**	**Conclusion concerning CTCs**	**Literature**
-	To assess the utility of CTCs in CRPC	CellSearch / ≥5 CTCs	CTC are the most accurate and independent predictor of OS in CRPC	(de Bono et al., 2008)
IMMC38	To assess CTC count as a prognostic factor for survival in CRPC	CellSearch / ≥5 CTCs	CTC counts and LDH concentration were the most predictive factors for survival	(Scher et al., 2009)
-	Characterization of ERG, AR and PTEN Gene Status in CTCs during abiraterone treatment	CellSearch + FISH / none	CTCs are malignant in origin and indicate that hormone-regulated expression of ERG persists in CRPC	(Attard et al., 2009)
-	Phase II multicenter study of abiraterone acetate plus prednisone therapy in CRPC	CellSearch / ≥5 CTCs	pre-therapy CTC counts are prognostic and their post-therapy change predicts survival	(Danila et al., 2010)
-	Use of the Adnatest® for detection of CTCs in CRPCs	AdnaTest / 0,1 ng/µl	CTCs were detected in 69 and 31% of patients before and during therapy; data on certain markers (EGFR) encourage future studies regarding treatment response	(Todenhöfer et al., 2012)
-	Randomized phase II trial of itraconazole	CellSearch / ≥5 CTCs	Thirty- two men had favorable baseline CTC counts; 96.9% of them retained favorable for 12 weeks; eight men had unfavorable baseline CTC counts five (62.5%) of them converted to favor able	(Antonarakis et al., 2013)
-	Molecular chaperone Hsp27 as a potential therapy target in	CellSearch / ≥5 CTCs	Hsp27 inhibitor decreased the number of CTCs in	(Shiota et al., 2013)

	CRPC, phase I		patients	
-	Phase II dose-ranging study of cabozantinib	CellSearch / ≥5 CTCs	86% patients with CTC decline had biochemical response in comparison with just 60% those who stayed unfavorable	(Lee et al. 2013)
-	Cabozantinib in a phase II nonrandomized expansion study	CellSearch / ≥5 CTCs	Cabozantitini improved CTC count, pain, bone scans and bone biomarker levels	(Smith et al. 2014)
SWOG S0421	Phase III trial of docetaxel with or without atrasentan	CellSearch / ≥5 CTCs	Baseline CTC counts were prognostic, and CTC rise at 3rd week was related to significantly worse OS	(Goldkorn et al. 2014)
TAK-700	Phase I/II trial of orteronel	CellSearch / ≥5 CTCs	22% CTC positive converted to a favorable CTC count and 57% retained a favorable CTC count at 12 weeks	(Dreicer et al., 2014)
-	The effect of PARP inhibitor olaparib in CRPC, phase II trial	CellSearch / ≥5 CTCs	29% patients had CTC reduction under the cut-off	(Mateo et al., 2015)
-	Phase II study of SB939, inhibitor of class histone deacetylases	CellSearch / ≥5 CTCs	CTC response (at 6 or 12 weeks) occurred in 9/14 evaluable patients	(Eigl et al., 2015)
COU-AA-301	abiraterone plus prednisone, phase III study	CellSearch / ≥5 CTCs	CTC number together with LDH level was shown to be a surrogate for survival	(Scher et al., 2015)
-	CTCs versus objective response assessment predicting survival in mCRPC	CellSearch / ≥5 CTCs	CTC counts are earlier and more sensitive predictor for survival and treatment response than current approaches. CTCs may provide complementary information toward individualized treatment strategies.	(M. Thalgott et al., 2015)
-	Long-term effects of CTCs in CRPC	CellSearch / ≥5 CTCs	At a median follow-up of 5 years, prognostic role of CTCs at baseline and during docetaxel chemotherapy was confirmed	(Verri et al., 2015)
-	Use of the AdnaTest® System for prognostic evaluation of CTCs in CRPC	AdnaTest / 0.15 ng/µl	CTCs were detected in 87% and 53% of patients before and during therapy; CTC presence during docetaxel therapy was associated with shorter OS	(Čapoun et al., 2016)

The prognostic power of CTCs was repeatedly established in clinical studies. A favorable change in CTC counts before treatment and during therapy proved to be associated with better disease control (Goldkorn et al., 2014; Scher et al., 2009; Verri et al., 2015). Despite their frequent use in clinical trials, CTCs had not been tested as a surrogate biomarker until the phase III clinical trial of abiraterone acetate. Although CTCs alone failed to prove their surrogacy, CTCs together with an increased level of LDH fulfilled the criteria. (Scher et al., 2015). However, another study confirmed CTCs to be a powerful prognostic and surrogate marker during docetaxel therapy (Figure 8). Concordance between CTC counts and RECIST and objective response evaluation was over 70% (M. Thalgott et al., 2015).

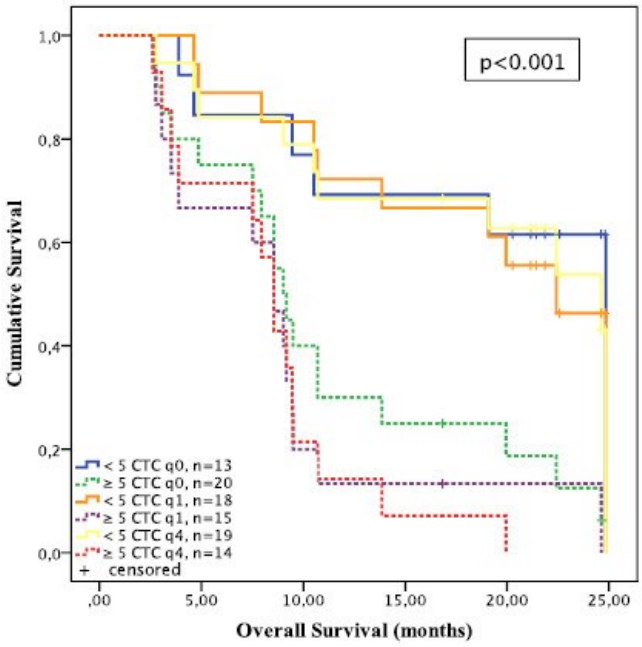


Figure 8: OS in favorable and unfavorable CTC group in different stages of docetaxel therapy (M. Thalgott et al., 2015). q0-before the therapy, q1-after the 1st docetaxel cycle, q4-after the 4th docetaxel cycle, q10-after the 10th docetaxel cycle

Since CTC determination enables the continuous monitoring of patients during studies, it has often been applied in clinical studies focused on introduction of new treatment. CTCs have proven useful as one of the evaluation criteria during clinical testing, such as in tests regarding the combination of abiraterone acetate and prednisone as the second-line treatment, the new bone protective therapy with Cabozantinib, intraconazole, an anticancer activity of the chaperon Hsp27 inhibitor, Orteronel and the DNA-repair mechanism inhibitor olaparib (Antonarakis et al., 2013; Danila et al., 2010; Dreicer et al., 2014; Lee et al., 2013; Mateo et al., 2015; Shiota et al., 2013; Smith et al., 2014).

Further knowledge on the therapeutics can be gained thanks to CTC analysis during clinical trials. A significant association has been observed between ERG rearrangement in tumor cells and the decline of PSA serum level in patients treated with abiraterone acetate. An ERG positive subpopulation of patients was thus shown to have heightened sensitivity to this treatment (Attard et al., 2009). However, the results of CTC testing can be contradictory to that of biochemical markers. When studying the use of the histon deacetylase inhibitor in CRPC, the PSA response was poor; however 64% of patients gained a favorable CTC count during the treatment (Eigl et al., 2015). Although the reason for the discrepancy is yet will to be assessed, CTCs definitely bring a new perspective to clinical studies of CRPC patients.

2 Aims of the study

Many new therapeutic possibilities are currently opening up for the treatment of patients suffering from CRPC. Together with the new therapies has come a need to determine and monitor therapy sensitivity, resistance and efficacy. However, a lack of serum markers as well as the impossibility of performing biopsies – on account of patients' advanced age and the bone localization of the disease - complicates the therapeutic decision-making in CRPC disease management.

CTCs are tumor cells released into the cancer patient blood from a tumor or metastasis. They can be collected from the blood and used as a liquid biopsy. CTCs are detected in the blood of the majority of CRPC patients. They have great potential to become a prognostic and therapy efficiency biomarker for CRPC patients.

The aim of this study was to explore the use of CTC-enriched samples obtained by the new method, AdnaTest (Qiagen, Germany), as a clinical biomarker as a part of liquid biopsy in the CRPC.

Major objectives:

- To implement the AdnaTest method and to evaluate its characteristics on patient samples.
- To correlate the results of CTC detection by the AdnaTest to the clinico-pathological characteristics of CRPC patients.
- To design and test a new multi-marker gene expression panel to monitor CTC character during CRPC therapy.
- To explore the use of CTC-enriched samples in high-throughput qPCR analysis
- To evaluate the semi-quantitative results of the AdnaTest by determining their correlation with the qPCR results measured on the BioMark platform.
- To investigate the gene expression in CTC-enriched samples and its relation to patient prognosis and therapy response with a special focus on the marker of anti-androgen therapy resistance, i.e. AR-V7.

3 Materials and Methods

3.1 Patient characteristics

Our study comprised 41 CRPC patients with evidence of metastatic disease. Diagnosis was made according to EAU Guidelines (Heidenreich et al., 2015). All patients had recently been diagnosed with CRPC at the time of their study enrolment and were indicated for docetaxel therapy in combination with prednisone. The performance status of all patients was two or less. sPSA level was measured before docetaxel therapy and before every docetaxel administration. The presence of metastases was assessed by scintigraphy and abdominal computer tomography (CT) scan. Biochemical and best response were assessed based on sPSA level and response evaluation criteria in solid tumors (RECIST) (Eisenhauer et al., 2009). Retrospective clinical data were obtained from the patients' charts.

Table 5: Study group characteristics.

	N	**%**
All patients	41	100%
Age (years);median (range)	74.5 (54.1-82.7)	
Gleason score		
≤ 7	24	59%
≥8	14	34%
Unknown	3	7%
Primary treatment		
Radical prostatectomy	10	24%
Radical radiotherapy	6	15%
Castration only	21	51%
Unknown	4	10%
Bone metastasis before Dtx	36	88%

≤ 3 bone lesions	9	22%
Multiple lesions	27	66%
Without bone metastasis	4	10%
Unknown	1	2%
Lymph node metastasis before Dtx	14	34%
Without lymph node metastasis	14	34%
Unknown	13	32%
sPSA at the time of PC diagnosis (ng/ml); median (range)	60 (3-782)	
sPSA at the time of CRPC diagnosis (ng/ml); median (range)	97 (2 - 770)	
sPSA before the 4th Dtx cycle (ng/ml); median (range)	54 (1 – 1243)	

Dtx: docetaxel chemotherapy, sPSA: prostate-specific antigen serum level

3.2 Immunomagnetic detection of circulating tumor cells

CTC presence was determined by the AdnaTest, a method using the immunomagnetic enrichment of CTCs followed by the immunomagnetic isolation of mRNA and the PCR detection of cancer-related genes. The commercially available AdnaTest Prostate Cancer Select and Detect kits (Qiagen, Germany) were used for the CTC analysis according to the manufacturer's protocols (Qiagen, 2017a, 2017b). The final PCR product detection and quantification was performed on a 2100 Bioanalyzer (Agilent Technologies, USA).

Two samples were analyzed from each patient; the first was drawn at the time of CRPC diagnosis and the second before the fourth docetaxel cycle. The CTC results were compared with the patients' clinic-pathological characteristics and other markers connected with the metastatic process (Chapter 4.2, Appendices 8.2 and 8.4).

3.2.1 Detection of circulating tumor cells by the AdnaTest method

The principle of the AdnaTest Prostate Cancer Select kit is based on the immunomagnetic enrichment of CTCs from whole (EDTA) blood by magnetic beads coated with antibodies against epithelial and tumor-associated antigens. The cells in the enriched fraction are lysed.

The AdnaTest Prostate Cancer Detect kit uses the immunomagnetic separation performed by beads coated with oligo-T oligonucleotides for messenger RNA (mRNA) isolation from the CTC-enriched lysate. Subsequently, mRNA is reverse transcribed to complementary DNA (cDNA). The cDNA was stored at -20°C until Multiplex-PCR and AR expression determination. The remaining cDNA was stored at -60°C to be used for further gene expression analysis. The Multiplex-PCR specific for epithelial/prostate-associated markers, i.e. epithelial growth factor receptor-EGFR, prostate-specific antigen-PSA and prostate-specific membrane antigen-PSMA, and a control gene, i.e. beta actin, occurs. A detailed description of this method can be found in Appendix 8.3 (Škereňová et al., 2016).

3.2.2 Additional detection of the androgen receptor in circulating tumor cells by the AdnaTest method

The AdnaTest ProstateCancer Detect kit newly enables the detection of AR in CTC-enriched samples. The cDNA samples from the study were retrospectively scanned for the detection of AR gene expression by Singleplex-PCR reaction according to the manufacturer's protocol (Qiagen, 2017b).

3.2.3 Final analysis of AdnaTest outcomes on the 2100 Bioanalyzer

The final AdnaTest result determination was made by on-a-chip capillary electrophoresis. The Agilent DNA 1000 Kit on a 2100 Bioanalyzer (Agilent Technologies, USA) was used for the PCR product detection and concentration determination. Samples were evaluated as CTC positive if the control PCR product, i.e. beta actin, was present and at least one of the monitored genes, i.e. EGFR, PSA, PSMA, was present in a concentration of 0.15 ng/ µl or higher. AR expression in CTCs was considered proven if the AR fragment (440bp) was present in a concentration of 0.15 ng/ µl or higher. PCR fragment measurement is described in detail in Appendix 8.3 (Škereňová et al., 2016). The results are presented in Chapter 4.2.1.

3.3 Circulating tumor cell visualization

The presence of CTCs in some samples was also assessed by optical and fluorescent microscopy.

3.3.1 Optical Microscopy

The binding of magnetic particles from the AdnaTest to PC cell line LNCaP cells in a spiking experiment and CTCs in patient samples was checked by using optical microscopy. A small amount (10 µl) of a solution containing magnetic beads from the AdnaTest Prostate Cancer Select kit was washed and added to one ml of either blood spiked with LNCaP cells or patient blood. The sample was then incubated for 30 minutes and washed three times in a magnetic holder using a phosphate buffer solution (PBS) to remove the blood cells. After the last washing the sample was resuspended in 10 µl of PBS and put on a microscopic slide. The presence of cells covered with magnetic particles was verified by optical microscope (Olympus BX51, Japan) and a photo was taken using a camera (Olympus DP 72, Japan). The picture of CTCs with magnetic beads is in Appendix 8.1 (Čapoun et al., 2014).

3.3.2 Fluorescent microscopy

A mononuclear layer from one ml of the patient's whole blood was isolated by gradient centrifugation (Histopaque®-1077, Sigma-Aldrich). Isolated cells were mounted on the microscopic slide. A dried sample was then fixed by fixation solution (5 ml 37% formaldehyde, 45 ml PBS) for 30 minutes. Subsequently, cell permeabilization occurred places (0,25 ml Tween 20, 49,75 ml H2O). The slides were washed three times in PBS for 5 minutes between each step. After careful removal of redundant liquid an antibody cocktail was put on the sample (2 µl of Anti-Cytokeratin pan−FITC, Sigma-Aldrich and 10 µl of Mouse Monoclonal to CD45 conjugated with phycoerythrin, Exbio, 188 µl of PBS). Samples were incubated in a humidity chamber over night. After final washing and drying a mounting medium and a cover slide were put on the microscope slide. Samples were checked by fluorescent microscope (Olympus BX51, Japan). The cells positive for pan-cytokeratin (stained green - FITC) were the cancer cells. Cells positive for CD 45 (stained red-phycoerythrin) were leukocytes. A blue staining of cell nuclei was present when mounting medium including 4',6-diamidino-2'-phenylindole (DAPI) was used. The results of fluorescent staining are presented in Chapters 4.1 and Appendix 8.1 (Čapoun et al., 2014).

3.4 Evaluation of circulating tumor cell detection by the AdnaTest method

3.4.1 Spiking experiment using prostate cancer cell line

To verify the ability of the AdnaTest to find cancer cells in the blood sample a spiking experiment with a PC cell line (LNCaP) was performed.

A stock solution with a concentration of one million LNCaP cells per ml was prepared by using a Bürker chamber and phosphate buffer solution (PBS). A series of 5 dilutions and one blank sample was prepared and spiked to the six different tubes each filled with five milliliters of the whole blood of a healthy donor. Samples with 0, 1, 10, 100, 1000 and 10000 cancer cells per ml of the blood were made. One ml of each sample was taken and used for fluorescent microscopy analysis. The samples were analyzed by using an AdnaTest Prostate Cancer kit according to the manufacturer's protocol. The results are presented in Chapter 4.1.1.

3.4.2 Determination of the method's characteristics on patient samples

The results of the size determination of four PCR products, i.e. beta actin, EGFR, PSA and PSMA, from the measurements of the first 33 CRPC patients performed during the first two years of the project, were used for method evaluation. The CTC determination was evaluated according to trueness, precision, repeatability, reproducibility and robustness. Additional experiments were performed on the 2100 Bioanalzyer which explored the repeatability, reproducibility and robustness of the concentration determination of the Agilent DNA 1000 kit. An overview is presented in Chapter 4.1.2 and further details can be found in Appendix 8.3 (Škereňová et al., 2016).

3.5 Analysis of gene expression on BioMark platform in circulating tumor cell-enriched and primary tumor samples

The cDNAs from CTC-enriched samples obtained during AdnaTest analysis were used as material for gene expression analysis. Comparisons were made of gene expression in primary tumor and CTC-enriched samples before and during cytotoxic treatment. Because of the limited amount of both cDNA sample types, an on-a-chip BioMark platform was chosen for the gene expression analysis.

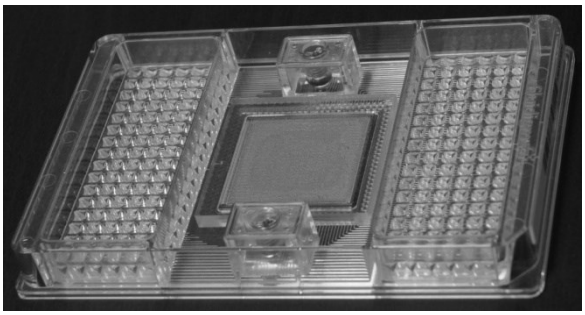


Figure 9: BioMark HD chip (Fluidigm®)

The microfluidic assay BioMark 96.96 Dynamic Array™ (Fluidigm, USA) enables analyses of up to 96 different genes in up to 96 samples on one chip (Figure 9). The BioMark assay minimizes sample volume and maximizes sample usage efficiency.

3.5.1 Gene expression panel formation

Since no gene expression panel directly focused on CTCs in CRPC has ever been established, our first goal was to choose genes whose expression would be determined in the analysis. Because of CTCs' unique potential as a liquid biopsy, the panel was composed of genes with an anticipated clinical practice application to investigate which of them could be successfully determined from the CTC-enriched cDNA samples. The emphasis was put on the genes which are already or may be used as therapy targets in PC. Other inclusion criteria were the role of the genes in therapy resistance, therapy efficacy and prognosis determination. The genes connected with the areas of high importance in CRPC disease management, i.e. reactivation of AR signaling, metastatic progression and causing an aggressive phenotype, were preferred.
Thorough literature search on PubMed and Web of Science as well as consultations with clinicians were performed to identify the best combination of genes for this specific purpose.
The number of genes in the panel was limited by the number of wells on the BioMark HD chip (Fluidigm®). Since the plan was for each gene to be measured in triplet and validation primers, IPC primers and negative controls had to be present on the chip, the final number of genes in the panel was set to 27.
The mechanisms of AR signaling pathway reactivation in CRPC patients and its influence on disease prognosis and therapy efficacy currently constitute one of the most discussed questions concerning PC. The cancer's ongoing progression despite androgen deprivation therapy could results from several proposed mechanisms. Since AR overexpression is one of the possible mechanisms, primers determining **AR** expression were added to the panel. The ligand-independent androgen receptor splice variant 7 (**AR-V7**) was suggested by

Antonarakis as another mechanism overcoming castration in CRPC as a therapy resistance marker of new AR-targeted therapies, i.e. abiraterone and enzalutamide. To have the opportunity to compare our results on the **AR** and **AR-V7** expression with Antonarakis' study, unique by studying gene expression in CTCs from CRPC patients, we used **AR** and **AR-V7** primers of the same design (Antonarakis et al., 2014).

Additionally, the activation of AR signaling in CRPC patients could be caused by the autocrine synthesis of testosterone and dihydrotestosterone (DHT). Their concentration in the prostate tissue of some PC patients has repeatedly been proven to be high despite castration and a low testosterone serum level. The key enzymes of steroidogenesis, e.i. **SRD5A1**, **AKR1C3** and **HSD3B2**, which have been found to be upregulated in CRPC, were added to the panel to find out if the autocrine activation of AR is present in CTCs (Adeniji et al., 2013; Chang et al., 2011; Stanbrough et al., 2006).

Since CTCs provide the real proof of an ongoing metastatic process, they are believed to express genes involved in tumor cell invasion. Genes involved in cell cycle control through the PI3K/AKT signaling pathway or NF-κB regulation system, i.e. **MT3**, **IGF1R**, **IL6**, **CXCL8** and **CD44**, prevent CTCs from apoptosis and enable their survival and proliferation. Genes playing a role in cell-cell interaction, i.e. **LGLAS1**, **CD44** and **BSG**, influence CTCs' motility and homing abilities (Hao et al., 2012; Hensler et al., 2016; Chun et al., 2009; Juang et al., 2013; Klein et al., 2002; Korski et al., 2014; Laderach et al., 2013; Mahon et al., 2011; Manna et al., 2013; Nguyen et al., 2014; Ni et al., 2014; Otsuka et al., 2013; Peng et al., 2016; Werner and Sarfstein, 2014).

Although new therapies have been introduced, therapy resistance remains a serious problem in CRPC disease management. Genes involved in therapy resistance, i.e. **FN1**, **TACSTD2**, **CLU**, **TRAP1**, **PTEN** and **PMEPA1**, have been identified. Some of these genes, i.e. **FN1**, **TACSTD2** and **PTEN**, are also known to introduce stem-like characteristics to cancer cells or to play a role in the epithelial-mesenchymal transition. Both of these mechanisms are important for CTC survival in the blood and they are connected with the more dangerous disease phenotype. Although the majority of chosen genes are believed to be overexpressed in cancer cells, **PTEN** and **PMEPA1** are tumor-suppressor genes and their expression should thus be downregulated (Altieri et al., 2012; Baron et al., 2006; Djeu and Wei, 2009; Chen et al., 2013; Leav et al., 2010; H. Li et al., 2015; Mahon et al., 2011; Punnoose et al., 2015; Qin et al., 2016; Shvartsur and Bonavida, 2015; Trerotola et al., 2013; Xie et al., 2014; Xu et al., 2003).

Additionally, genes specific for PC cells, i.e. **AMACR**, **EGFR**, **ERBB2**, **KLK3** and **FOLH1**, were added to ensure the presence of prostate cells in cDNA from CTC-enriched samples. Moreover, most of these genes, i.e. **EGFR**, **ERBB2**, **KLK3** and **FOLH1**, have the potential to become prognostic markers in CRPC. They also serve as therapy resistance markers, i.e. **ERBB2** and **EGFR**, and therapy targets, i.e. **EGFR**, **ERBB2** and **FOLH1** (Artibani, 2012; Box et al., 2016; Day et al., 2017; Dijkstra et al., 2014; Gao et al., 2016; Gorges et al., 2016; Chen et al., 2013; D. T. Miyamoto et al., 2012; Okegawa et al., 2016; Ristau et al., 2014; Whang et al., 2013; Yates et al., 2012; Zehentner et al., 2006).

Finally, four reference genes known for their stable expression in cells, i.e. **ACT**, **HPRT1**, **TUBB** and **UCB**, were added to the gene panel to be used for the gene expression evaluation. A complete list of genes monitored in the CTC-CRPC gene expression panel can be found in Table 6.

Table 6: Panel of 27 genes selected for the expression analysis of CTCs in CRPC patients.

Assay name	Official full name	Summary
AR FN	androgen receptor	AR signaling represents a crucial mechanism in PC progression. Although AR signaling was believed to be inactivated in CRPC, it was proven that AR is overexpressed in some CRPC patients. The status of AR expression is an important factor for treatment choice and can aid understanding of the mechanism of disease progression in an individual patient (Yuan et al., 2014).
AR-V7	androgen receptor splice variant 7	AR-V7 lacks the androgen binding domain and causes the continual activation of AR signaling. The presence of AR-V7 has been proven in CTCs of CRPC patients and it is believed to be connected with worse prognosis, disease progression and resistance to AR-targeted therapeutics.(Antonarakis et al., 2014)
SRD5A1	steroid-5-alpha-reductase, alpha polypeptide 1	SRD5A1 is a leading enzyme converting testosterone to DHT which has stronger affinity to AR. The overexpression of SRD5A1 has been identified as one of the mechanisms leading to CRPC development. High conversion of residual testosterone to DHT enables reactivation of AR signaling and disease progression despite castration. SRD5A1 represent also a therapy target for dutasteride which can prevent ADT resistance (Chang et al., 2011; Lunardi et al., 2013).
AKR1C3	aldo-keto reductase family 1, member C3	AKR1C3 is a key enzyme of steroidogenesis in the prostate. It catalyzes the reduction of androgen precursors to testosterone. AKR1C3 can cause AR signaling reactivation in CRPC patients by synthesizing testosterone from alternative substrates. Its overexpression is connected with the development of CRPC. AKR1C3 inhibitors are intensively studied as potential therapeutics in CRPC (Adeniji et al., 2013)
HSD3B2	hydroxy-delta-5-steroid dehydrogenase	HSD3B2 is a crucial enzyme in steroid synthesis converting dehydroepiandrosterone (DHEA) to testosterone precursors. Its overexpression has been observed in CRPC and is believed, together with AKR1CA and SRD5A1a, to be part of the autocrine

		activation of the AR signaling pathway. HSD3B2 is a possible target of sunitinib therapy (Dutt and Gao, 2009; Kroiss et al., 2011; Stanbrough et al., 2006).
MT3	metallothionein 3	MT3 is known to be highly expressed in prostate tissue probably due to a high zinc content. MT3 has been connected with the invasion, proliferation and cytotoxic therapy resistance of cancer cells. However conflicting reports on the connection between MT3 expression and androgen signaling in PC have been published and the role of MT3 expression in CRPC has yet have to be established (Juang et al., 2013; Otsuka et al., 2013).
IGF1R	insulin-like growth factor 1 receptor	IGF1R is highly overexpressed in many malignancies including PC. It protects cells from apoptosis and plays a role in malignant transformation. IGF1R has also been connected with the increased motility of tumor cells and was chosen as a target for PC immunotherapy (Hensler et al., 2016; Werner and Sarfstein, 2014).
IL6	interleukin 6 (interferon, beta 2)	IL6 induces the proliferation of prostate cells. It has been suggested that IL6 plays a major role in the development of the castration-resistant form of PC. IL6 helps tumor cells overcome androgen deprivation therapy, possibly by inducing the expression of enzymes involved in androgen synthesis. IL6 expression is connected with the more aggressive disease phenotype and the development of bone metastases. IL6 serves as a target for immunotherapeutic siltuximab (Hudes et al., 2013; Chun et al., 2009; Nguyen et al., 2014).
CXCL8	chemokine (C-X-C motif) ligand 8, Interleukin 8	The expression of CXCL8 is increased in metastatic and CRPC. It plays a role in cancer cell proliferation, survival, migration and the induction of angiogenesis. CXCL8 may also contribute to the chemotherapy and androgen deprivation therapy resistance of cancer cells (Mahon et al., 2011; Manna et al., 2013).
CD44	CD44 molecule	The CD44 molecule is a glycoprotein involved in cell-cell interactions, cell adhesion and migration. It has been identified as a stem cell marker in PC cells. CD44 is connected to cell invasion, proliferation, epithelial-mesenchymal transition and chemoresistance. These roles makes CD44 an optimal future therapy target (Hao et al., 2012; Korski et al., 2014; Ni et al., 2014; Orian-Rousseau, 2010).
LGALS1	lectin, galactoside-binding,soluble 1, galectin 1	LGALS1 has been found to be the most commonly expressed galectin in PC. Its expression has been found to be upregulated especially during disease progression. Galectin 1's role in cell-cell interactions and signaling is thought to be important in tumor cell survival, motility and immune-escape. Galectin 1 is also involved in tumor angiogenesis. LGALS1 inhibition is a new potential target of cancer therapy (Astorgues-Xerri et al., 2014; Laderach et al., 2013).
BSG	Basigin, CD147	BSG is one of the immunoglobulins and is known as an extracellular matrix metalloproteinase inducer. Its high expression has been found in many tumors including PC. BSG expression was detected in the majority of tumor cells from prostate and breast cancer micrometastases. Its expression seems to correlate with metastases development. BSG targeting leads to limitation of metastasis and increases chemosensitivity (Hao et al., 2012; Klein et al., 2002; Peng et al., 2016).
FN1	fibronectin 1	FN1 is a glycoprotein involved in cell adhesion and migration including the metastatic process. FN1 is known to be overexpressed in cancer cells and is a marker of epithelial-mesenchymal transition

		in CTCs. Moreover, it has been connected with a resistance to docetaxel and tested as a possible cancer therapy target (Baron et al., 2006; Chen et al., 2013; Kaspar et al., 2006; Qin et al., 2016).
TACSTD2	tumor-associated calcium signal transducer 2	TACSTD2 is overexpressed in many cancers. Its overexpression provides tumor cells with stem cell-like characteristics. TACSTD2 is also connected with therapy resistance. It helps androgen-sensitive PC cells to overcome taxane-based chemotherapy. TACSTD2 is a potential prognostic and therapy efficacy marker in PC. Its role as a therapy target has also been investigated (Shvartsur and Bonavida, 2015; Trerotola et al., 2013; Xie et al., 2014).
CLU	clusterin	CLU is a stress-induced chaperone which plays an important role in cancer therapy resistance. Its higher expression in PC cells is known to be a reaction to chemotherapeutic treatment. An anti-sense oligonucleotide against CLU mRNA is being tested as an additional treatment to docetaxel to prevent the development of therapy resistance. CLU could also be responsible for resistance to other drugs by blocking apoptotic signals in cells (Djeu and Wei, 2009; Mahon et al., 2011).
TRAP1	TNF receptor-associated protein 1	TRAP1 is a chaperon from the mitochondrial-specific heat shock protein 90 (HSP90) family. TRAP1 is overexpressed in PC in comparison with healthy prostate tissue. It inhibits mitochondrial apoptosis, regulates oxidative stress response and is connected with chemotherapy resistance acquisition. Its silencing in cancer cells increases their sensitivity to chemotherapy. TRAP1 is also a new potential target for cancer therapy (Altieri et al., 2012; Leav et al., 2010).
PTEN	phosphatase and tensin homolog	PTEN is one of the most frequently inactivated tumour suppressor genes in cancer. PTEN negatively regulates the PI3K–AKT–mTOR pathway responsible for cell survival, proliferation and multidrug resistance. Loss of PTEN regulation leads to the expression of multiple proto-oncogenes and growth factors stimulating cancer progression. PTEN status is an important molecular characteristic of individual tumor and it has also already been determined from CTCs (Mahon et al., 2011; Punnoose et al., 2015).
PMEPA1	prostate transmembrane protein, androgen induced 1	PMEPA1 is an androgen inducible gene highly expressed in prostate but downregulated in PC cells. PMEPA1 is a negative regulator of PC cell growth, expression of AR and transforming-growth factor beta signaling. Decreased expression of PMEPA1 is connected with lower PTEN expression, cancer progression and increased resistance to AR inhibitors. PMEPA1 monitoring could serve for ADT efficacy determination (H. Li et al., 2015; Xu et al., 2003).
AMACR	alpha-methylacyl-CoA racemase	AMACR is widely used as a PC marker in biopsy samples. AMACR is known to be overexpressed in PC in contrast with normal tissue. AMACR has also been used as a CTC marker in PC patients. Its higher expression is associated with disease progression. AMACR expression is related to TMPRSS2-ERG gene rearrangement and could thus serve as prognostic marker in PC. AMACR silencing also presents a treatment option in PC (Artibani, 2012; Box et al., 2016; Takahara et al., 2009; Zehentner et al., 2006).
EGFR	epidermal growth factor	EGFR is a member of protein kinase superfamily. EGFR expression was found to be higher in advanced, metastatic and androgen-

	receptor	independent prostate tumors of prostate. Its presence correlates with worse prognosis and is currently connected with androgen signaling reactivation in CRPC. EGFR expression has successfully been measured in CTCs and may serve as a prognostic and therapy efficacy marker. It is a therapeutic target for epidermal growth factor inhibitors, e.g. lapatinib (Chen et al., 2013; Okegawa et al., 2016; Whang et al., 2013).
ERBB2	erb-b2 receptor tyrosine kinase 2, human epidermal growth factor receptor 2	ERBB2 gene encodes a member of the epidermal growth factor receptor family of receptor tyrosine kinases also known as HER2. ERBB2 is overexpressed during PC progression and CRPC development. ERBB2 appears to be able to restore AR signaling by a ligand-independent mechanism. It may play a role in antiandrogen resistance and bone metastases development. It is a therapeutic target for epidermal growth factor inhibitors, e.g. lapatinib (Day et al., 2017; Gao et al., 2016; Whang et al., 2013).
KLK3	kallikrein-3, prostate specific antigen	KLK3 also known as prostate specific antigen (PSA) is a protease secreted by prostate cells. Its serum level is the major marker in PC. However, KLK3 expression in CTCs has been shown to correlate with sPSA. KLK3 expression may serve as a marker of CTCs' prostate origin as well as a PC prognostic marker. Moreover, KLK3 expression is driven by AR and thus can be a marker of ongoing AR signaling (Dijkstra et al., 2014; D. T. Miyamoto et al., 2012; Yates et al., 2012).
FOLH1	folate hydrolase,prostate-specific membrane antigen 1 (PSMA)	FOLH1 is also known as prostate specific membrane antigen (PSMA) and was one of the first identified PC-specific antigens. FOLH1 expression increases from benign prostatic hyperplasia to high-grade PC. Its expression is higher in progressing and androgen-independent PC. FLOH1 expression is also connected with bone and lymph node metastases development. It is a possible therapeutic target and prognostic marker in CRPC (Gorges et al., 2016; Ristau et al., 2014).
ACT	actin, beta	Reference gene
HPRT1	hypoxanthine phosphoribosyl transferase 1	Reference gene
TUBB	tubulin, beta 2A class Iia	Reference gene
UCB	ubiquitin C	Reference gene

3.5.2 Probe assay design

A probe assay was chosen for the gene expression analysis in order to maximize its specificity. The assay includes not only two primers (forward and reverse) for sequence specification but also a probe which binds between the primers. The probe is an oligonucleotide with a fluorescent (FAM) label and a quencher (ZEN). When primers are elongated during the PCR reaction, the probe is disintegrated by polymerase and fluorescent labels are liberated from the quenchers. The subsequent increase of fluorescence is proportional to the increase in the number of specific PCR fragments. This system ensures

that the quantum of fluorescence corresponds, in contrast with intercalating fluorescent dyes e.g. SYBR green, only with the amplification of a specific gene. The highly-sensitive double-quenched probe assay, PrimeTime ZEN^{TM} (IDT, USA, Coralville), which reduces background fluorescence, was used for the measurement (Figure 10).

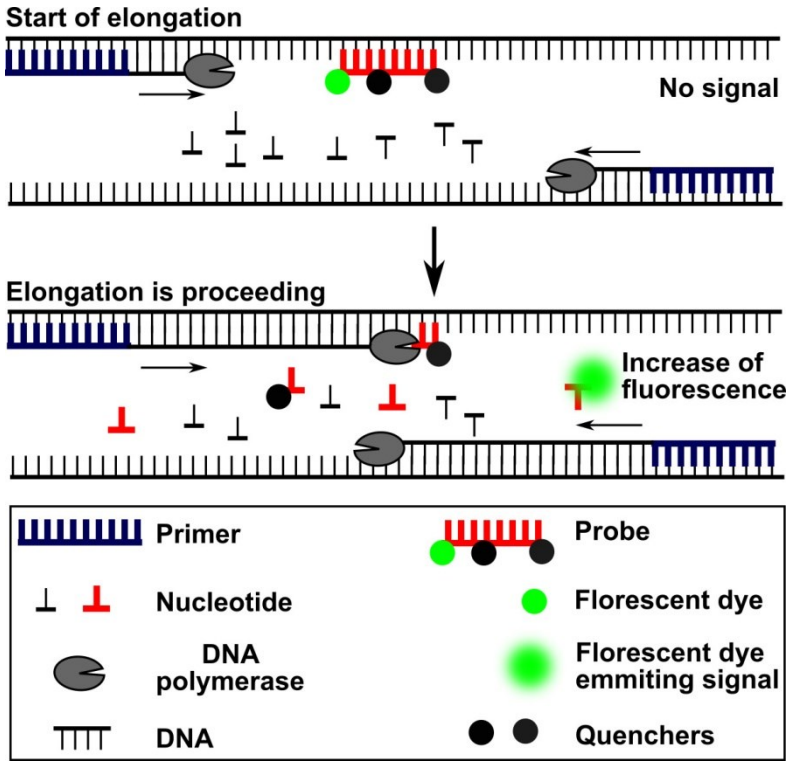


Figure 10: Principle of Probe assay in qPCR. Adapted from (IDT, 2017)

Sixteen primer-probe sets from 27 genes in the panel were obtained from a commercial source, the GrandPerformance Probe Based CTC Assay Panel (Tataa Biocentrer, Sweden). The remaining 11 primer-probe sets were designed *de-novo* by using an NCBI/Primer-BLAST tool (NCBI, 2016) and Probe Designer with the following criteria: i) optimal annealing temperature 60°C; ii) uses exons which span introns longer than 500 bp-for mRNA use; iii) if several gene splice variants, target all if possible - not applicable for AR-V7; iv) use short amplicons - for FFPE use. The sequences of the designed primers and probes are presented in Appendix 8.5 (Škereňová et al., submitted 2017). The designed primers and probes were ordered from IDT (Integrated DNA Technologies, USA).

3.5.2.1 Primer and probe validation

All newly - synthesized primers and subsequently the primer-probe sets were checked for their efficiency in qPCR techniques using a TATAA SYBR® Grandmaster®Mix (Tataa

Biocenter, Sweden) and LightCycler 480 II (Roche, Switzerland). Primer dimer presence was checked in control samples with no template. If no signal was detected after 45 cycles it was assumed no primer dimers had been generated. Genome DNA (gDNA) amplification was checked, because the presence of some gDNA in the sample could interfere with result analysis. The gDNA was used as a template with each primer and the signal was measured. If any exponential increase (Cq) occurred before 25 cycles the primer was assumed to be interacting with gDNA. The primers' specificity was checked by measuring the melting curves of the PCR products. The detailed description of primer and probe validation can be found in Appendix 8.5 (Škereňová et al., submitted 2017).

3.5.3 Preamplification testing

Because of the anticipated low quantity of cDNA in both sample types, i.e. CTC-enriched samples from the AdnaTest analysis and FFPE primary tumor samples, a preamplification step was introduced. Preamplification increases the amount of targeted DNA in the samples whilst the proportion of individual genes stays the same. A preamplification step was performed upon the mixture of all 27 primer-probe sets.

The threshold cycle number (Cq) of preamplified cDNA was compared with the Cq of non-preamplified material and the preamplification efficacy was calculated. The preamplification efficacy was evaluated as sufficient within the range of 80-105%. Non-template preamplification controls (NTC) were run in duplicate for each primer-probe set to ensure that no interaction between primers generating unspecific results took place. If a signal was detected, the unspecific product of the measured primer-probe pair and some other pair was generated during PCR analysis. The preamplification results are presented in Chapter 4.3.2 and Appendix 8.5 (Škereňová et al., submitted 2017).

After a successful preamplification evaluation, all the samples chosen for gene expression analysis were preamplified by using the same protocol.

3.5.4 CTC-enriched and primary tumor tissue samples selection and preparation

Two types of samples were measured in the gene expression assay. Firstly, the cDNA samples collected during the CTC measurement by the AdnaTest were used. Samples of cDNA from the first and the second CTC measurement were available from each patient except those who missed the second blood draw. The samples contained cDNA transcribed from mRNA from

the lysed CTC-enriched fraction. They provide the information about gene expression in CTCs. The detailed description of CTC-enriched sample preparation is in Chapter 3.2.1. All samples were stored at -60°C until they were slowly thawed before the preamplification and gene expression analysis.

Secondly, cDNA samples isolated from FFPE primary tumor samples were used to compare gene expression between primary tumor and CTC-enriched samples.

3.5.4.1 Primary tumor tissue sample preparation

To compare gene expression between CTC-enriched samples and a fixed primary tumor, samples of primary tumor of 31 patients from the study were obtained from the department of Pathology, General University Hospital in Prague. FFPE samples of tumor tissue were acquired via a needle biopsy or during an operation (prostatectomy). Samples from ten patients were not found, either because they were used up during the tumor characterization or because of the biopsy occurred in another medical centre.

A FFPE RNA Purification Kit (Norgen Biotech, Canada) was used for RNA isolation from primary tumor tissue samples (Norgen Biotek, 2015). The kit enables partial reversion of formalin-caused RNA modifications thus resulting in the high yield and quality of the obtained RNA. The isolation took place on a column on a chloroform-free basis. From 5 to 25 mg, containing as much tissue as possible, were cut from FFPE blocks and homogenized in 1.5 ml tubes according to the protocol. Deparaffinization was performed by washing the samples repeatedly with xylene and ethanol. Subsequently, a protease K was used, releasing nucleic acids from the proteins. Lysate was transferred to a separation column and spun down. Nucleic acids were bound to the membrane and DNA was removed by introducing an RNA purifying step including DNase I. The obtained RNA was washed on the column, eluted by nuclease-free water and stored at -20°C.

From one to five RNA samples were isolated from each of the 31 patients. RNA concentration and purity was determined by NanoDrop (Thermo Scientific, USA) and Qbit (Thermo Scientific, USA). One sample from each patient - that with the highest concentration and better purity - was chosen for subsequent analysis.

All RNA samples were transcribed to cDNA by reverse transcription (RT) by using TATAA Grandscript Supermix (TATAA Biocenter, Sweden). Four µl of supermix was mixed with samples and nuclease-free water to a final volume of 20 µl and a final RNA concentration of 30 or 12.5 ng/ µl for samples with high or low RNA concentration, respectively. Samples

were put in a thermocycler with a program: 25°C, 5 min; 42°C, 30 min; 85°C, 5 min. The synthesized cDNA was stored at -20°C.

3.5.4.2 Final selection of samples for gene expression analysis

Samples of 32 out of the 41 patients enrolled in the study were analyzed for gene expression. The primary tumor and both samples from CTC detection were available for 14 patients. Twelve patients had samples from a primary tumor and the first CTC analysis. Four patients had samples from both CTC draws but no primary tumor sample. Two patients had two samples of primary tumor (from needle biopsy and from prostatectomy) plus a sample from the first CTC analysis.

Except for two testing samples, cDNAs from CTC sampling evaluated by the AdnaTest as CTC negative samples were not used in the expression analysis. Sample 73C was evaluated as CTC negative, however, AR expression was detected in this sample. Sample 75C was measured as a negative control. Samples with a poor quality of RNA or a low amount of sample were removed from the analysis. The number of samples on the chip was decreased from 96 to 78 because blank samples and positive and negative controls had to be put on the chip. For this reason, some samples which did not have another sample for the same patient for comparison were removed from the analysis. The list of the samples used and those which were excluded from the gene expression analysis can be found in Table 7, together with the reason for sample removal.

Table 7: List of the samples loaded on the chip. Patients excluded completely from the analysis are marked in red. Samples marked in red were excluded from the analysis (the reason is marked by a symbol: *CTC negative sample; †no sample for comparison; ‡poor quality of RNA or low quantity of sample).

Patient Nr.	FFPE	1st CTC	2nd CTC	Patient Nr.	FFPE	1st CTC	2nd CTC	Patient Nr.	FFPE	1st CTC	2nd CTC
1	1P	1C	8C‡	**15**	15P	24C	29C*	**29**	29P	54C*	59C*
2	2P	2C	10C*	**16**	16P	43C	51C	**30**	30P‡	55C	56C
3	3P	3C	18C*	**17**	17P	28C	36C*	**31**	31P†	57C*	62C*
4	4P	4C	14C	**18**	no	30C	37C	**32**	32P	58C	63C
5	5P	5C	22C	**19**	19P	31C	-	**33**	33P	60C	64C
6	6P	6C	15C	**20**	$20P_{1,2}$	33C	38C*	**34**	no	61C	66C
7	7P	7C	13C	**21**	21P	35C	46C*	**35**	no	65C†	67C*
8	8P	9C	26C	**22**	no	39C	45C	**36**	no	68C†	-
9	9P	11C	20C*	**23**	no	40C†	48C*	**37**	no	69C*	-
10	10P	12C	23C	**24**	24P†	41C*	-	**38**	38P	70C	72C
11	11P	16C*	27C*	**25**	no	42C†	53C*	**39**	39P	71C	73C*
12	$12P_{1,2}$	17C	32C*	**26**	26P	44C	49C	**40**	40P	74C	76C
13	no	19C†	-	**27**	27P	47C	50C	**41**	41P	75C*	-
14	14P	21C	34C*	**28**	no	52C†	-				

3.5.5 Gene expression measurement on the BioMark platform

A 96-well chip for the BioMark platform (Fluidigm, USA) was used for a gene expression assay (Figure 9). The chip was primed by injecting control line fluid according to the manufacturer's instructions (Fluidigm, 2016) followed by its placement in the integrated fluidic circuit controller and running the Prime (136x) script.

Preamplified samples were diluted 10 times with TE buffer. Each sample (1 µl) was mixed with SG loading buffer (0.25 µl; Fluidigm, USA), TATAA probe GM mix (2.5 µl; Tataa Biocenter, Sweden) and nuclease free water (1.25 µl). The mixture (5 µl) was loaded on a chip to the left sample zone according to a prepared scheme by using a multichannel pipette. Each primer-probe mixture (2 µl) was mixed with 2x assay loading reagent (2.5 µl; Fluidigm, USA) and nuclease free water (0.5 µl). The mixture (5 µl) was loaded on a chip to the right primer zone in triplets according to a prepared scheme by using a multichannel pipette. The chip was placed in the integrated fluidic circuit controller and a script Load Mix (136x) was run to pump all samples and primers through the microfluidic channels to the wells in the centre of the chip. In each of 136 wells each sample was mixed with each primer.

The loaded chip was placed in the BioMark™ machine and 136 qPCRs ran at the same time. The program contained 45 cycles of three steps: 95°C 30s; 45x 95°C 5s, 60°C 30s. The chip was scanned for the fluorescence signal in every cycle and amplification curves were measure for each well. By using Data Collection Software (Fluidigm, USA) data were visualized and Cq values were checked and manually corrected if required (see Chapter 4.3.3).

The presence of the monitored PCR fragments in CRPC patients was described and correlated with gene expression determined on the BioMark platform (see Chapters 4.2.1, and Appendix 8.5).

The results were correlated between the primary tumor and CTC-enriched samples (see Chapter 4.3.4) as well as between CTC-enriched samples from the various therapy time points (see Chapter 4.3.5). The relative gene expression of monitored genes was correlated with patient survival and a therapy response (see Chapter 4.3.5).

3.6 Statistics

Statistical analyses concerning patients' clinical data and their relationship with CTC status were assessed by standard statistical tests performed using SAS 9.4 software (Cary, NC, USA). A detailed description of the tests is available in Appendices 8.2, 8.3 and 8.4 (Čapoun et al., 2016; Skerenova et al., 2017; Škereňová et al., 2016).

Gene expression data were analyzed by using the SAS 9.4 and GENEX (version 6) programs. Logistic regression and the Chi-squared test were used for the data in binary form. The relative expression data were analyzed by a mixed model. Spearman's and Pearson tests were used for correlation analyses (see Appendix 8.5) (Škereňová et al., submitted 2017).

4 Results and discussion

The AdnaTest is one of the methods developed for the detection of CTCs. In this thesis, the complex use of this technique as a liquid biopsy for the detection and further characterization of CTCs in CRPC patients is studied. The method in combination with high-throughput qPCR analysis is also evaluated for its potential in CRPC therapeutic decision-making.

The principle of the method was tested by using a PC cell line and its characteristics, determined on patients' samples, were compared with the manufacturer's information (Chapter 4.1).

The detection of CTCs by the AdnaTest method was investigated as a marker of CRPC patient prognosis and therapy efficacy. The role of CTCs in the metastatic process of advanced cancer patients was also studied (Chapter 4.2).

A panel of 27 genes related to the therapeutic decision-making in CRPC patients was established, designed and tested (Chapters 4.3.1, 4.3.2 and 4.3.3).

The use of CTC-enriched samples as a material for gene expression analysis on the BioMark platform was analyzed (Chapters 4.3.4. and 4.3.5.1).

The association between the AdnaTest results and the relative gene expression determined on the BioMark platform was assessed (Chapter 4.3.5.2).

Gene expression changes in CTC-enriched samples during the docetaxel therapy were studied and discussed in the context of current literature. The impact of the monitored genes on patient survival and therapy response was investigated (Chapters 4.3.5.3, 4.3.5.4 and 4.3.5.5).

4.1 Evaluation of circulating tumor cell detection by the AdnaTest method

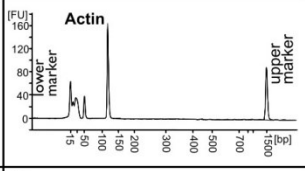
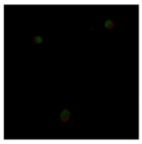
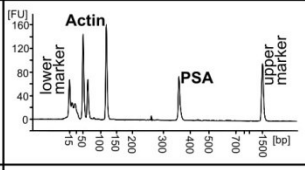
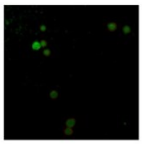
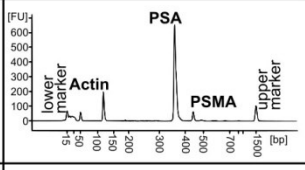
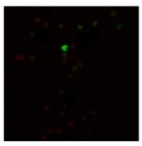
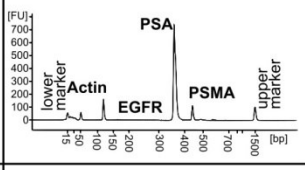
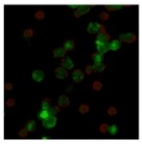
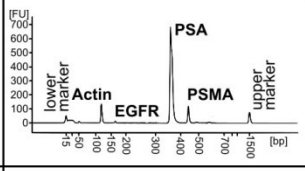
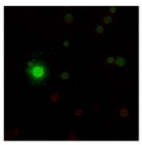
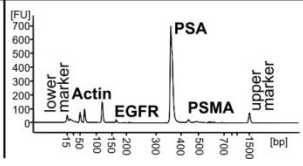
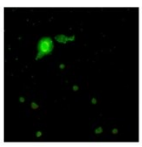
The principle of the AdnaTest was verified by a using cancer cell line followed by the evaluation of its characteristics in patient samples.

4.1.1 Spiking experiment using prostate cancer cell line

A series of six different dilutions of cells from the LNCaP PC cell line spiked to the blood of a healthy donor were measured by the AdnaTest (Table 8). Only the sample without PC cells was found negative. Samples with an increasing concentration of PC cells showed increasing concentration of monitored PCR fragments. The only exception was the PSMA fragment in

the last sample in which the lower final concentration of the PCR fragment was probably caused by the exhaustion of PCR reagents because of the high content of PC cells. Fluorescent staining of the samples verified the presence of the LNCaP tumor cells. The tumor cells are recognized according to cytokeratin expression (strong green signal) and the lack of expression of CD45 (red edges) which is expressed by leukocytes.

Table 8: AdnaTest results for six different serial dilutions of an LNCaP PC cell line in the blood of a healthy donor. Fluorescent staining proved tumor cells (green staining of pancytocertin) as well as leukocytes (red staining of CD45) to be present in the samples.

LNCaP cell concentration	Electropherogram	PCR fragment concentration [ng/µl]		Immuno-fluorescent staining
blank sample		Actin EGFR PSA PSMA	6.9 0.0 0.0 0.0	
1 cell/ml		Actin EGFR PSA PSMA	5.9 0.0 2.1 0.0	
10 cells/ml		Actin EGFR PSA PSMA	7.1 0.0 21.2 1.4	
100 cells/ml		Actin EGFR PSA PSMA	5.6 0.1 27.2 2.5	
1 000 cells/ml		Actin EGFR PSA PSMA	6.3 0.5 37.2 3.6	
10 000 cells/ml		Actin EGFR PSA PSMA	7.8 0.8 39.2 1.0	

EGFR-epithelial growth factor receptor, PSA-prostate specific antigen, PSMA-prostate specific membrane antigen.

This verification of the semi-quantitative character of the AdnaTest compensates its inability to determine the exact number of CTCs in the samples. Until recently, the majority of studies

concerning CTCs used a threshold of 5 CTCs for result evaluation. However, the analysis of CTCs as a continuous variable has been demonstrated as an alternative approach to CTC evaluation (Daniel C Danila et al., 2007; Mark Thalgott et al., 2015). This analytical method can be applied to the semi-quantitative results of the AdnaTest as well as to the CTC counts.
The AdnaTest proved the ability to differentiate between blood samples with and without PC cells. The semi-quantitative character of PCR fragment concentration results was confirmed (Table 8).

4.1.2 Evaluation of AdnaTest method characteristics on patient samples

The evaluation of the AdnaTest's characteristics was made simultaneously with the measurement of samples for a clinical study (see Chapter 4.2.2). The results are summarized in Table 9.

Table 9: Summary of method characteristics based on the results obtained from two years of CTC research (PCR fragment size measurement) and additional experiments (PCR fragment concentration measurement). Adapted from (Škereňová et al., 2016).

	Purpose of testing		Sample type	Number of measured samples	Average RSD
Obtained characteristics for PCR fragment size determination	Precision		Positive control	31	**under 2%**
	Trueness			31	**under 3%**
	Precision		Patient samples	101*	**under 2%**
	Trueness			101	**under 3%**
Obtained characteristics for PCR fragment concentration determination	Repeatability		Positive control	12	**15%**
	Robustness	Sample volume		3×8	**17±2%**
		Marker mix volume		3×4	**9±3%**
	Inter Multi-PCR Repeatability			3×3	**19±10%**
	Inter Multi-PCR Reproducibility			6×1	**37±16%**
	Repeatability		Patient samples	3×3	**17±3%**
	Reproducibility for PCR product storage**			1×1×12	**17±14%**
	Reproducibility for cDNA storage**			1×1×1×12	**40±20%**

RSD-relative standard deviation; * Not all samples from patients contained all monitored fragments. Only Actin as a control fragment was present in each measurement. Consequently, the number of measurements is different for each fragment: N_{Actin}=101, N_{EGFR}=15, N_{PSA}=69, N_{PSMA}=31. The number of measurements is higher than the total number of patients since some of the samples were measured several times;** Samples were stored for 10 months at -20°C.

The characteristics based on patient sample measurements were satisfactory and correspond very well with the manufacturer's data, which were based on the measurement of a standardized material. Further details can be found in Appendix 8.3 (Škereňová et al., 2016).

4.2 Circulating tumor cell detection by the AdnaTest method in castration-resistant prostate cancer patients

The determination of CTCs in CRPC patients by the AdnaTest was evaluated and put in context with the patients' clinical characteristics. Moreover, the prognostic value of CTCs in CRPC patients and the role of CTCs in the metastatic process were studied.

4.2.1 Detection of circulating tumor cells by the AdnaTest according to the presence of monitored PCR fragments

The results of 41 CRPC patients measured by the AdnaTest are depicted in Table 10, including the additional AR test.

Eight out of 41 patients did not undergo the second CTC test during therapy. Five patients were lost to follow up, one patient died of cardiac failure, one patient interrupted chemotherapy after the first cycle and one patient did not finish the third chemotherapy cycle before the end of the study.

Table 10: Evaluation of CTCs in 41 CRPC patients by the AdnaTest at the time of diagnosis and during chemotherapy together with the concentration of monitored PCR fragments of tumor-associated genes (EGFR, PSA, PSMA, AR). PCR fragments evaluated as positive are gray (light for fundamental AdnaTest monitored PCR fragments, darker for an additional AR PCR fragment).

Patient number	At the time of CRPC diagnosis					After the third cycle of docetaxel				
	CTC Evaluation	EGFR (ng/µl)	PSA (ng/µl)	PSMA (ng/µl)	AR (ng/µl)	CTC Evaluation	EGFR (ng/µl)	PSA (ng/µl)	PSMA (ng/µl)	AR (ng/µl)
1	**positive**	0	14.0	0.5	4.3	**positive**	0	4.3	0.1	0.2
2	**positive**	0	1.8	0	0	negative	0	0	0	0
3	**positive**	0	4.2	0	0	negative	0	0	0	0
4	**positive**	0.3	9.2	0	1.7	**positive**	0	1.8	0	0
5	**positive**	0	15.4	0.3	0	**positive**	0	10.3	0	0.6
6	**positive**	0	0.7	0	0	**positive**	0	0.2	0	0
7	**positive**	0	5.3	0.2	0.3	**positive**	0	1.7	0	0.2
8	**positive**	0.5	41.8	11.3	25.1	**positive**	0	16.1	7.4	0.6
9	**positive**	0	14.4	0.9	0.3	negative	0	0	0	0
10	**positive**	0	35.1	0.5	18.9	**positive**	0.2	39.3	0.5	16.1
11	negative	0	0	0	0	negative	0	0	0	0
12	**positive**	0.1	17.6	1.1	12.2	negative	0	0	0	0
13	**positive**	0.1	15.6	0.7	3.9	-	-	-	-	-
14	**positive**	0	7.2	0	3.6	negative	0	0.1	0	0
15	**positive**	0	9.3	0.8	5.3	negative	0	0	0	0
16	**positive**	0	0.6	0	0	**positive**	0.2	0.1	0	0
17	**positive**	0	24.9	0.6	19.4	negative	0	0	0	0
18	**positive**	0	23.9	6.0	3	**positive**	0	2.9	1.1	0.3
19	**positive**	0	18.9	1.8	0	-	-	-	-	-
20	**positive**	0	0.2	0	0	negative	0	0	0	0

21	**positive**	0	0	0.3	0	negative	0	0	0	0
22	**positive**	0.1	10.8	1.1	2.5	**positive**	0	2.1	0	0.3
23	**positive**	0	5.2	0.4	2.5	-	-	-	-	-
24	negative	0	0	0	0.5	-	-	-	-	-
25	**positive**	0	12.0	0.3	0.9	negative	0	0	0	0
26	**positive**	0	3.4	0	4.2	**positive**	0	1.0	0	0
27	**positive**	0.1	6.6	0.6	5.7	**positive**	0	3.0	0.1	2.2
28	**positive**	0.2	17.2	0.2	18.2	-	-	-	-	-
29	negative	0	0.1	0	1.5	negative	0	0	0	0
30	**positive**	0	0.6	0	5.8	**positive**	0	0.4	0	0.5
31	negative	0	0	0	0	negative	0	0	0	0
32	**positive**	0.1	7.1	0.2	6.5	**positive**	0.5	15.7	2.6	13.8
33	**positive**	0	1.2	0	1.0	**positive**	0	4.2	0.1	0.6
34	**positive**	0.1	14.7	3.0	10.2	**positive**	0	3.6	0.1	2.21
35	**positive**	0	1.2	0.2	0	negative	0	0	0	0
36	**positive**	0	0.3	0	0	-	-	-	-	-
37	negative	0	0	0	0	-	-	-	-	-
38	**positive**	0	19.6	8.2	14.9	**positive**	0.1	3.7	0.1	6.1
39	**positive**	0.5	0	0	0	negative	0	0	0	0
40	**positive**	0	7.2	0.2	0.2	**positive**	0	9.4	0.4	0.2
41	negative	0	0	0	0.3	-	-	-	-	-

EGFR-epithelial growth factor receptor, PSA-prostate specific antigen, PSMA-prostate specific membrane antigen, AR-androgen receptor

Consistent with similar studies, the majority (85%) of the patients in our study were CTC positive at the time of CRPC diagnosis. After the third cycle of docetaxel therapy, only 45% of the patients remained CTC positive in our study compared with 61% in Thalgot's study and 31% in Todenhöfer's study (M. Thalgott et al., 2015; Todenhöfer et al., 2012). However, the difference may have been caused by differences in study designs e.g. detection method, sampling protocol.

None of six CTC negative patients in our group became CTC positive during the therapy. However, three of those patients did not undergo the second test.

Owing to the principle of the test we cannot say for sure if a decrease in PCR fragment concentrations is caused by a decreased expression of monitored genes in CTCs or by a decreased number of CTCs in the sample. However, based on the results from other studies we can presume that CTC counts decreased during the therapy in the majority of samples, causing the measured decrease in PCR fragment concentrations (de Bono et al., 2008; Olmos et al., 2009; Scher et al., 2009; Thalgott et al., 2013b).

The frequency of detection of individual PCR fragments was similar at the time of CRPC diagnosis and during therapy, with the only exception being PSMA (Table 11). The frequency of PCR fragments is slightly higher but comparable with the only other study made on a similar group of patients (Todenhöfer et al., 2012). However, Todenhöfer's study comprised only 16 patients, which may be the cause of the difference.

A new option to determine the AR status in PC has recently been added to the AdnaTest and was measured in our study (Table 11). The change in AR expression may be crucial for patient prognosis and sensitivity to AR-targeted therapies (Yuan et al., 2014).

Table 11: Frequency of AdnaTest monitored PCR fragments in CTC positive patients at the time of diagnosis and during docetaxel therapy.

	Frequency of individual gene detection in CTC positive patients	
	At the time of CRPC diagnosis (N=35)	**After the third cycle of docetaxel** (N=18)
EGFR	17%	17%
PSA	94%	94%
PSMA	66%	28%
AR	69%	78%

At the time of CRPC diagnosis, 70% of AR positive samples were positive for both PSA and PSMA. Although the frequency of AR positivity increased during the therapy, PSA and PSMA "coexpression" remained only in 36% of AR positive samples. The expression of PSA in PC is a result of the AR signaling pathway. In contrast, the upregulation of PSMA expression in PC was shown to be induced by antiandrogen therapy, i.e. by the downregulation of the AR pathway (Evans et al., 2011; Leversha et al., 2009; Murga et al., 2015).

The AdnaTest cannot identify if the presence of AR, PSA and PSMA in one sample is a result of their coexpression in all CTCs or if each of them comes from a different CTC subpopulation or if it is a combination of both. However, other studies have presented the intra-patient heterogeneity of CTCs and we can presume, that the subpopulation of CTCs with ongoing AR signaling and PSA expression exists alongside the subpopulation of PSMA-expressing CTCs. The decrease in PSMA frequency of detection may be caused by the docetaxel therapy, which may more greatly affect this CTC subpopulation (Gorges et al., 2016; Todenhöfer et al., 2012). Regardless of the origin of individual PCR fragments, the knowledge of their status in each patient and the opportunity to follow up their development

during therapy represents a valuable tool in cancer monitoring of particular importance in targeted therapy, in which the presence or absence of a specific gene product may help in therapeutic decision-making (David T Miyamoto et al., 2012; Nakazawa et al., 2015).

Interestingly, the expression of AR was also detected in three CTC negative patients at the time of CRPC diagnosis (Table 10). A subpopulation of CTCs which does not contain any of three PCR fragments fundamentally monitored by the AdnaTest may exist and similar results concerning EMT markers have already been published (Todenhöfer et al., 2012). A dedicated study is needed to establish the status of these samples: according to current guidelines, samples positive only for AR are evaluated as CTC negative.

To conclude, CTCs detected by the AdnaTest vanished in half of the patients and the concentration of monitored PCR fragments decreased in the majority of samples during docetaxel therapy. The frequency of detection of monitored PCR fragments remained stable during the therapy, except for PSMA, the frequency of detection of which decreased. The frequent detection of AR together with the high concentrations of PSA suggests ongoing AR signaling. The status of the samples with the sole presence of AR has yet to be established.

4.2.2 Circulating tumor cells as a prognostic marker

The prognostic role of CTCs in CRPC patients is discussed in Chapter 6.2 (Čapoun et al., 2016). However, the relevance of the studied characteristics became clearer after a prolonged follow-up period. The results of the final analysis with updated data are summarized below.

The median follow-up of 41 patients before the final data analysis was 23.5 months. Patient characteristics are presented in the materials and methods section in Chapter 3.1.

The CTC count had already been proven to be associated with CRPC patient survival, therapy response and metastases presence (Bitting et al., 2015; Goldkorn et al., 2014; M. Thalgott et al., 2015). However, in this study, a similar role of CTCs detected by the AdnaTest was verified. The detection of CTCs before and during the therapy was associated with worse disease specific survival (DSS) of the patients (Figure 11). None of the patients without CTCs at the time of CRPC diagnoses had disease progression in comparison with 20% of CTC positive patients. Similarly, there was a significant difference in bone metastases presence between the patients with and without CTCs during therapy ($p=0.033$). The sPSA level was significantly higher in the patients with CTCs during therapy (Figure 12). Although the role of sPSA level as a marker in CRPC is full of contradictions, a higher sPSA level is connected with worse therapy response and metastatic progression (Armstrong et al., 2012).

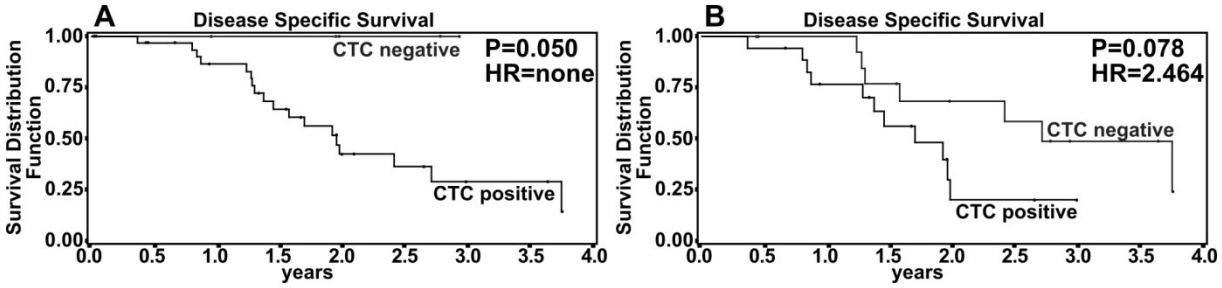


Figure 11: DSS of CTC positive vs. CTC negative patients A) at the time of CRPC diagnosis and B) during docetaxel therapy. CTC-circuating tumor cells, HR-hazard ratio.

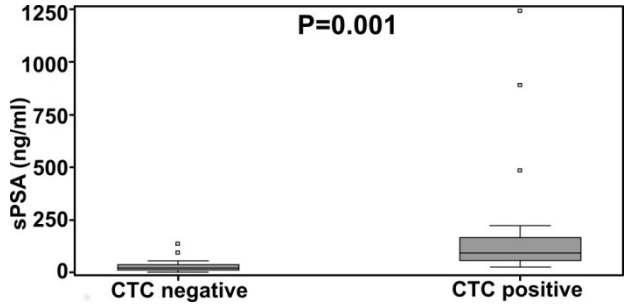


Figure 12: sPSA level measured during docetaxel therapy is significantly higher in the CTC positive patients.

As demonstrated above, simple CTC positivity plays a role in patient prognosis, but the detection of monitored PCR fragments can also serve as an indicator of prognosis. An expression of EGFR is a known negative prognostic marker in CRPC patients. EGFR determined in CTCs by the AdnaTest at the time of CRPC diagnosis resulted in a worse DSS (Figure 13A) (Todenhöfer et al., 2012). Similarly, the DSS was significantly worse for the AR positive patients at the time of CRPC diagnosis (45.0 vs. 20.4 months, p=0.011, HR=5.586) as well as during therapy (45.0 vs. 17.5 months, p=0.003, HR=4.501). Only one patient out of 33 followed became AR positive during of docetaxel therapy in comparison with nine patients who became AR negative (Table 10). Despite the fact that the patients in the study did not undergo any AR-targeted therapy, the change in AR status was associated with a significantly different DSS (Figure 13B).

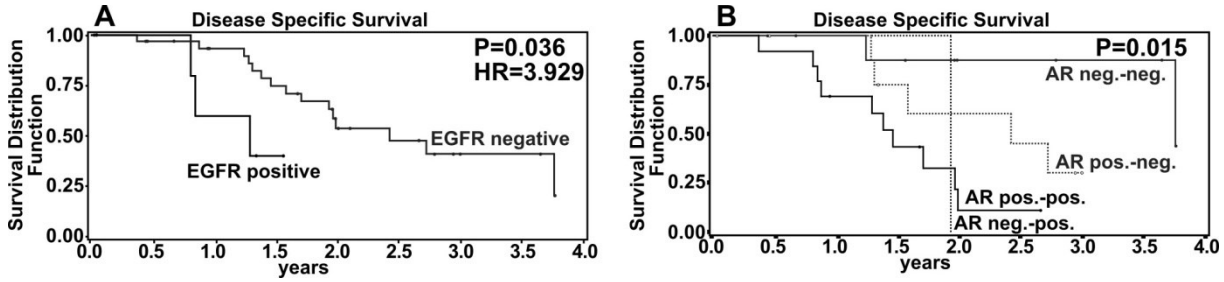


Figure 13: Presence of PCR fragments determined by the AdnaTest may predict worse DSS in CRPC patients: A) EGFR positivity determined at the time of diagnosis, B) AR status development at the time of diagnosis and during the therapy. EGFR-epidermal growth factor receptor, HR-hazard ratio, AR-androgen receptor, pos.-positive, neg. –negative.

The AR positive patients showed a higher level of sPSA in both measurements (Figure 14A, B). The patients without AR during therapy experienced a decrease in sPSA between the measurements, indicating a positive response to the therapy. In contrast, the relative change of sPSA in AR positive patients was significantly worse, suggesting a worse therapy response (Figure 14C).

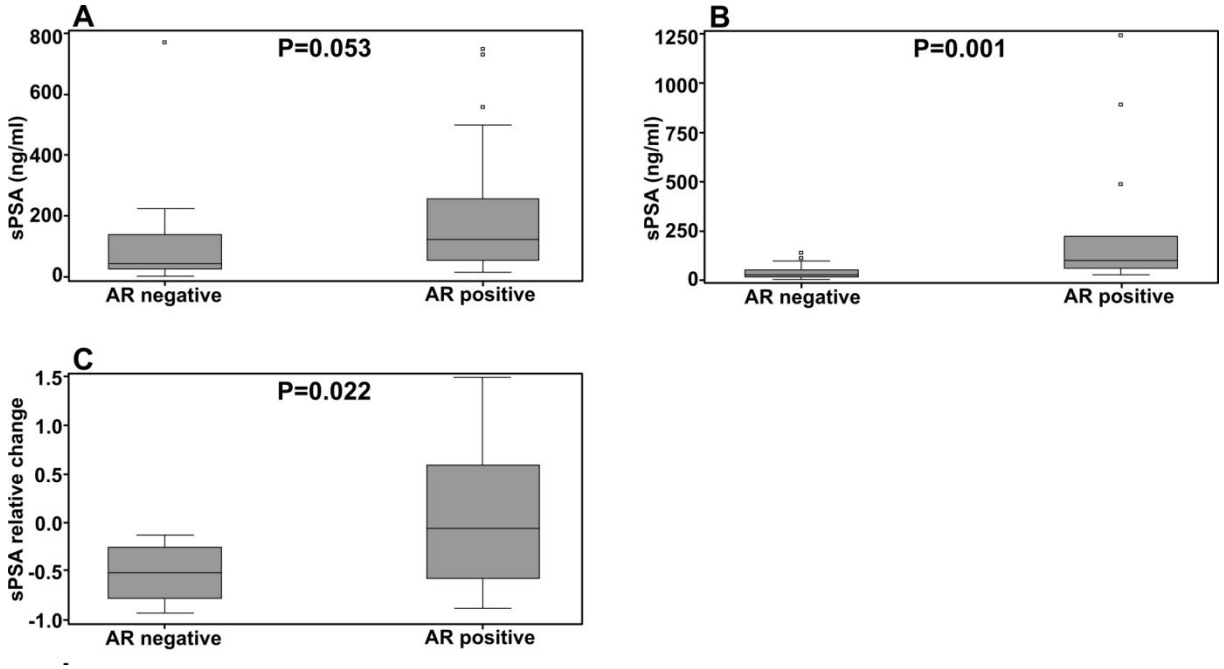


Figure 14: AR positivity determined by the AdnaTest is connected with a higher sPSA level both, A) at the time of CRPC diagnosis and B) during docetaxel therapy. C) The relative change of sPSA is significantly better in AR negative patients during therapy. PSA-prostate specific antigen. AR-androgen receptor

In conclusion, CTCs detected by the AdnaTest may predict patient' survival and are associated with the sPSA level. Moreover, CRPC patients can be stratified into groups with different survival and therapy response according to the detection of PCR fragments by the AdnaTest, i.e. EGFR and AR. The AR status follow-up may be beneficial for patients indicated for AR-targeted therapy (Antonarakis et al., 2014).

4.2.3 Circulating tumor cells and metastatic serum marker levels in advanced cancer patients

The role of CTCs in the metastatic process was investigated as part of a larger study in order to solve specific issues in advanced cancer patients. The levels of matrix metalloproteinase 2 (MMP2), matrix metalloproteinase 9 (MMP9) and vascular endothelial growth factor (VEGF), plus CTC presence were monitored in 24 CRPC patients and in 44 HER2 positive breast cancer (HER2 BC) patients to find markers of therapy efficacy and proceeding metastatic process, respectively (Skerenova et al., 2017).

In contrast with a high CTC positivity rate in CRPC patients (Table 10), CTCs were found only in 17% of HER2 BC patients. This reflects the effect of HER2 targeted therapy on CTCs (Cristofanilli et al., 2004) which ideally could also be achieved in CRPC patients by applying new targeted therapies. Currently, the persistence of CTCs in patient blood during therapy is connected with shorter survival (Skerenova et al., 2017).

No correlation was found between serum marker levels and CTCs in CRPC patients but the MMP2 serum level was significantly higher in CTC positive HER2 BC patients (Figure 15). The MMP2 serum level plays an important role in metastases formation. Consistent with other studies, the higher MMP2 serum level was connected with the presence of bone and central nervous system metastases (De Giorgi et al., 2010; Peeters et al., 2014).

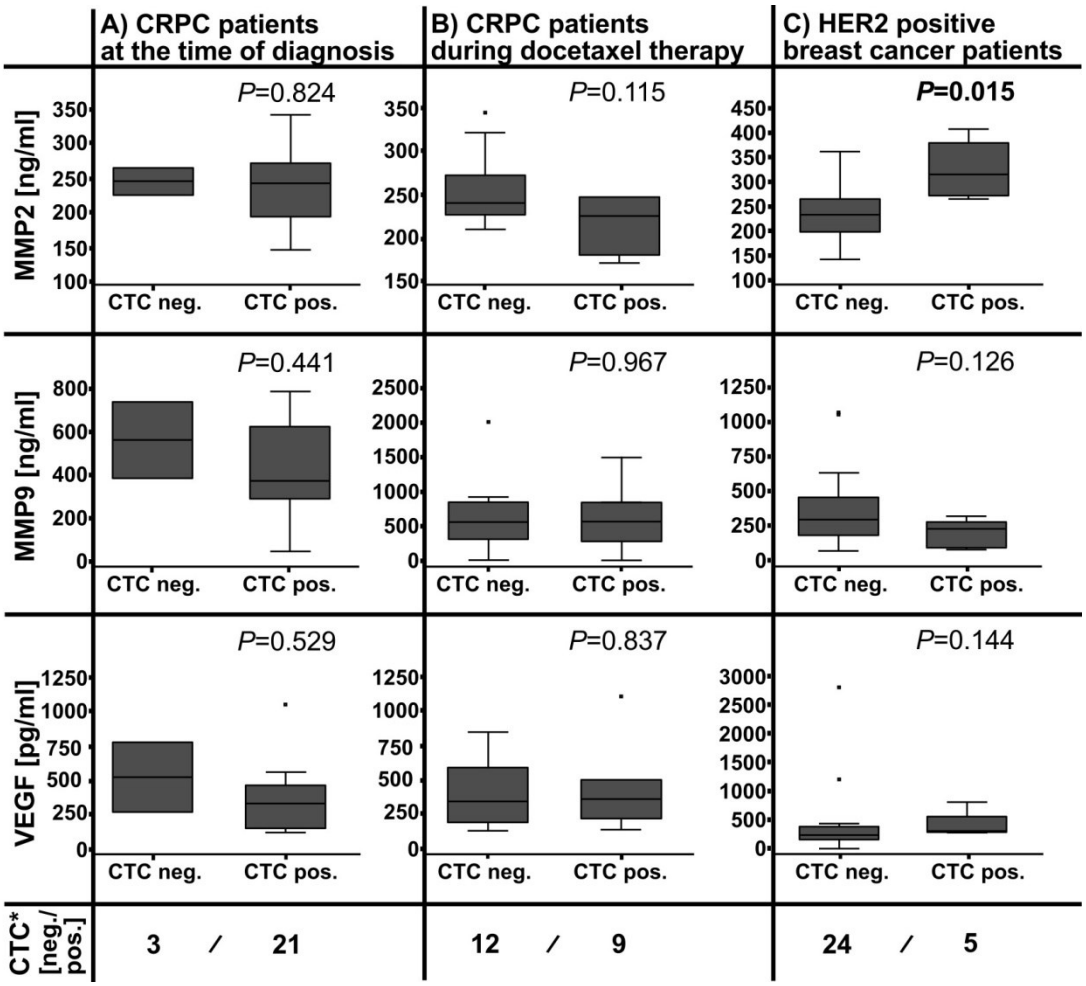


Figure 15: The comparison of MMP-2, MMP-9 and VEGF serum levels in CTC positive and negative patients: A) CRPC patients at the time of diagnosis, B) CRPC patients during docetaxel therapy and C) HER2 positive breast cancer patients undergoing targeted palliative treatment. Statistically significant differences are in bold (P<0.05). Adapted from (Skerenova et al., 2017).

MMP2-matrix metalloproteinase 2, MMP9-matrix metalloproteinase 9, VEGF-vascular endothelial growth factor, HER2-human epidermal growth factor receptor 2, neg.-negative, pos-positive. *Number of CTC measurements may differ from number of serum marker measurements. For details see Appendix 8.4 (Skerenova et al., 2017).

To summarize, the detection of CTCs by the AdnaTest was proven as a useful CTC detection method in advanced breast and PC. Despite the preliminary style of the study and limited number of patients, the results verified the important role of CTCs in metastatic progression and their indisputable place amongst cancer markers; see Chapter 6.4 (Skerenova et al., 2017).

4.3 Gene expression analysis of circulating tumor cell-enriched samples on the BioMark platform

To further explore the use of CTC-enriched samples as a liquid biopsy material, a multi-marker qPCR gene expression assay was designed. Genes were chosen according to their role as therapy targets and therapy resistance markers in CRPC. The expression of monitored genes was compared between CTC-enriched samples and FFPE samples of a primary tumor. Changes in gene expression during the therapy as well as the impact of individual gene expression on patient survival and therapy response were investigated.

4.3.1 Design and validation of primers and probes

The gene expression panel, focused on therapeutic decision-making in CRPC based on gene expression in CTCs, consists of 27 genes (Table 6). For eleven of these genes the primer-probe sets had to be designed *de novo*. The designed sets were tested for their specificity and for their use in the BioMark 96.96 Dynamic Array™ (Fluidigm, USA).

4.3.1.1 Primer and probe design

The final sequences of eleven newly-designed primer-probe sets for the gene expression panel are presented in Table 12.

Table 12: Primer and probe sequences designed for CTC-CRPC gene expression panel.

Assay Name	Forward Primer	Reverse Primer	Probe	Amplicon length
HS3DB2	GGGAGCAATGAGTATGTGG	AAACAGAAGATGCTGGAGAG	CCCTCTTCTGGGTCACGCTAGAATCA	83
MT3	GTGGCTCCTGCACCT	GTCCTTGGCACACTTCTC	CTGCTGCTCCTGCTGCCCTG	113
IL6	AAAAGTCCTGATCCAGTTCC	GGCTGGCATTTGTGGTT	TCTAGATGCAATAACCACCCCTGACC	83
CXCL8	TGCAGCTCTGTGTGAAG	GTGGAAAGGTTTGGAGTATG	TCTAAGTTCTTTAGCACTCCTTGGCAA	88
LGALS1	AAACCTGGAGAGTGCCTT	CACAGGTTGTTGCTGTCTT	AGCCACCTCGCCTCGCACTC	92
BSG	TACTCCTGCGTCTTCCTC	GACTTCACAGCCTTCACTC	ACGGCCAACATCCAGCTCCAC	83
FN1	CATAAAGGGCAACCAAGAGA	GGTGTTGTAAGGTGGAATAGA	CCAAAGCCACTGGAGTCTTTACCACAC	85
CLU	AGCAACCTAGAAGAAGCCA	GGTCTCATTGCACACTCC	AGCTTTGTCTCTGATTCCCTGGTCTCA	102
TRAP1	AAAGTCCTCATCCAGACCAA	TCAGGGGAATGTCCTCAC	CCACCACACCTCGGATGAAGCG	91

PMEPA 1	TGCACGGTCCTTC ATC	GCCACAGGCATCCTT C	CAGGGCATCTTCTCTCCTC CGC	77
AMACR	AAGCGGTCGGAT GTG	AGCTAACCGGCAGAA G	CCAAATCCACTCAGCCTGG CATAAAT	150

4.3.1.2 Primer validation

The amplification efficacy for each primer pair was calculated from the dependence of Cq on template concentration. The charts, together with the amplification efficacy, are depicted in Figure 16. The efficacy of all the designed primer pairs was within the recommended range (80-105%). The melting curve analysis proved that all primer pairs generated just one PCR product (Figure 17). The specificity of the primers is verified by the presence of just one peak, i.e. one melting temperature of all PCR products, in each assay. No other products, e.g. primer dimers, were detected. A weak amplification of genomic DNA was observed during the validation of the primers CXCL8, TRAP1 and HSD3B2.

Primers which could not be validated by using the mixed cDNA sample from tumor cell lines on account of low expression levels were validated by using their own PCR products as a template to verify their characteristics. The results of the validation of these primers are presented in Section B, Figures 16 and 17.

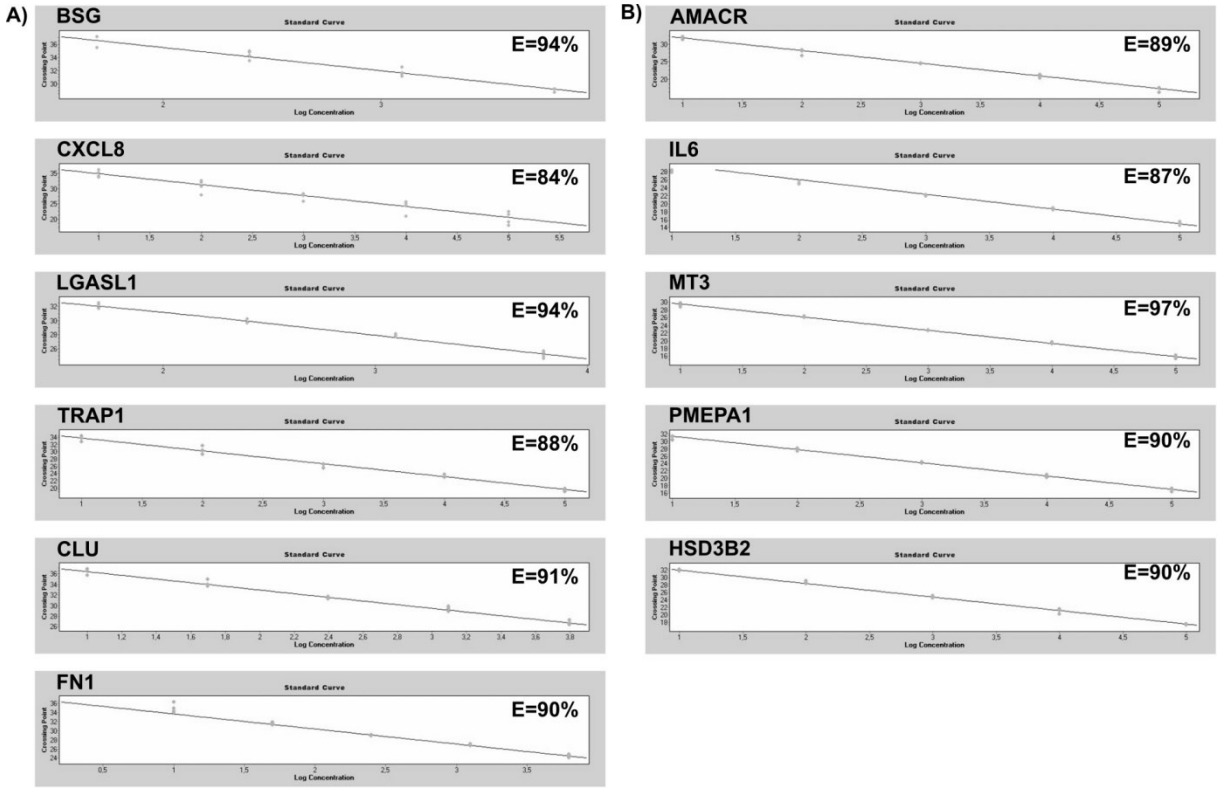


Figure 16: Standard curves of the Cq dependence on log of template concentration. Dilution experimentation was performed to determine the amplification efficacy of primers A) with cDNA from tumor cell lines B) with PCR product as a template.

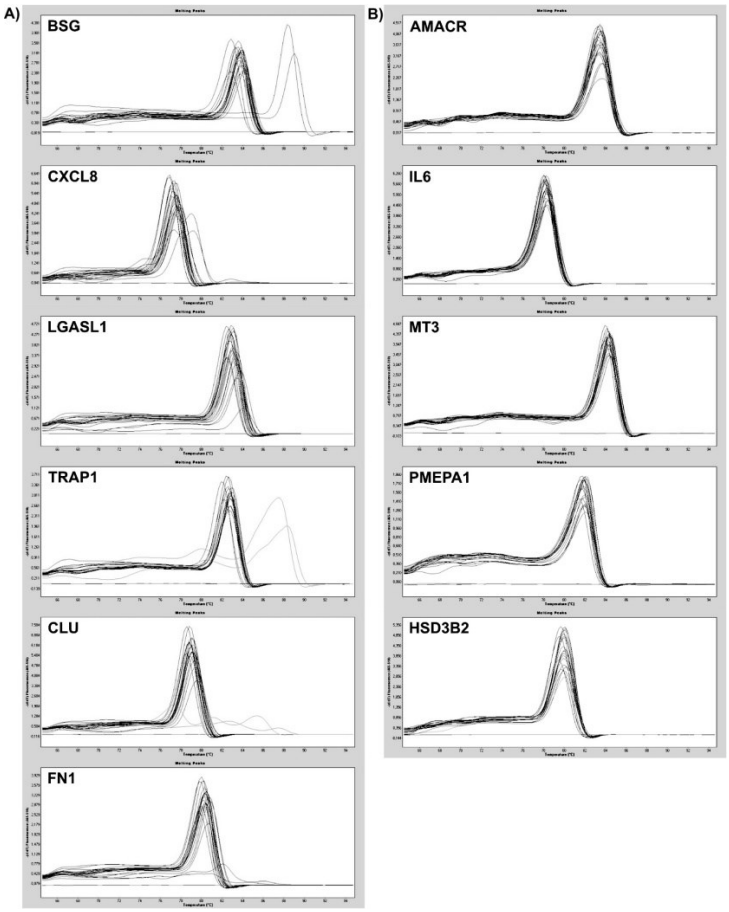


Figure 17: Melting curves (the dependence of a fluorescent signal on temperature) of the PCR products of the designed primers A) with cDNA from tumor cell lines B) with PCR product as a template.

To conclude, all the designed primer pairs were shown to be specific for the sequence and efficient enough for use in the assay (see Appendix 8.5) (Škereňová et al., submitted 2017).

4.3.1.3 Probe validation

After primer validation, primer-probe set validation was performed. The amplification efficacy of all the probe assays was within the recommended range of 80-105%. The dependence of the Cq on template concentration together with efficacy values are depicted in Figure 18. BSG and CXCL8 primer-probe sets showed week interaction with genomic DNA

(gDNA) which has to be taken in account in result evaluation. The efficacy of some primer-probe sets - AMACR, IL6, PMEPA1, TRAP1 and HSD3B2 - had to be tested on PCR products because of their poor expression in cDNA from common tumor cell lines.

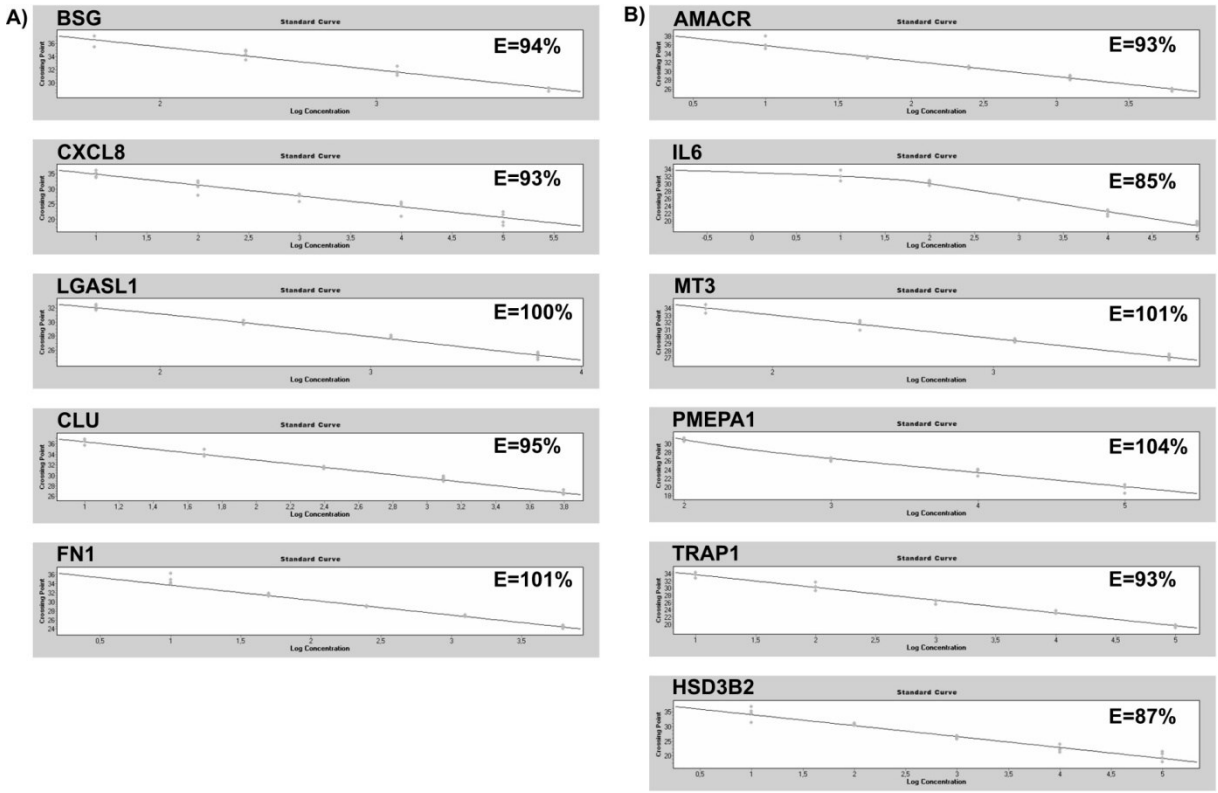


Figure 18: Standard curves of the Cq dependence on log of template concentration. Dilution experimentation was performed to determine the amplification efficacy of primer-probes sets A) with cDNA from tumor cell lines B) with PCR product as a template.

To summarize, the validation of the designed primer-probe sets was successful and the characteristics of the sets were sufficient for their use in the gene expression assay (see Appendix 8.5) (Škereňová et al., submitted 2017).

4.3.2 Preamplification testing

The results of the analysis of the correlation between the preamplified and non-preamplified samples were satisfactory (Figure 19). Differences present in the region of higher Cq were most probably caused by small sampling errors which were multiplied by DNA amplification. The preamplification run according to this protocol should not influence the proportional representation of monitored genes in a sample.

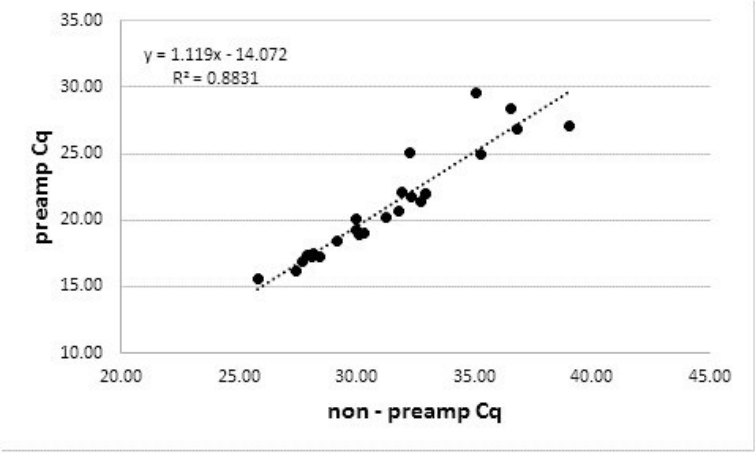


Figure 19: Correlation of Cq of preamplified and non-preamplified cDNA samples by using primer mixture of 27 primer-probe sets from the CTC-CRPC gene expression panel.

The preamplification efficacy was calculated for each primer-probe set and is depicted in Figure 20. Most of the genes fulfil the efficacy criteria. PMEPA1, HSD3B2 and AMACR showed a low preamplification efficacy and should be analysed with caution. KLK3 and AR-V7 cannot be analyzed because of their low expression in non-preamplified samples.

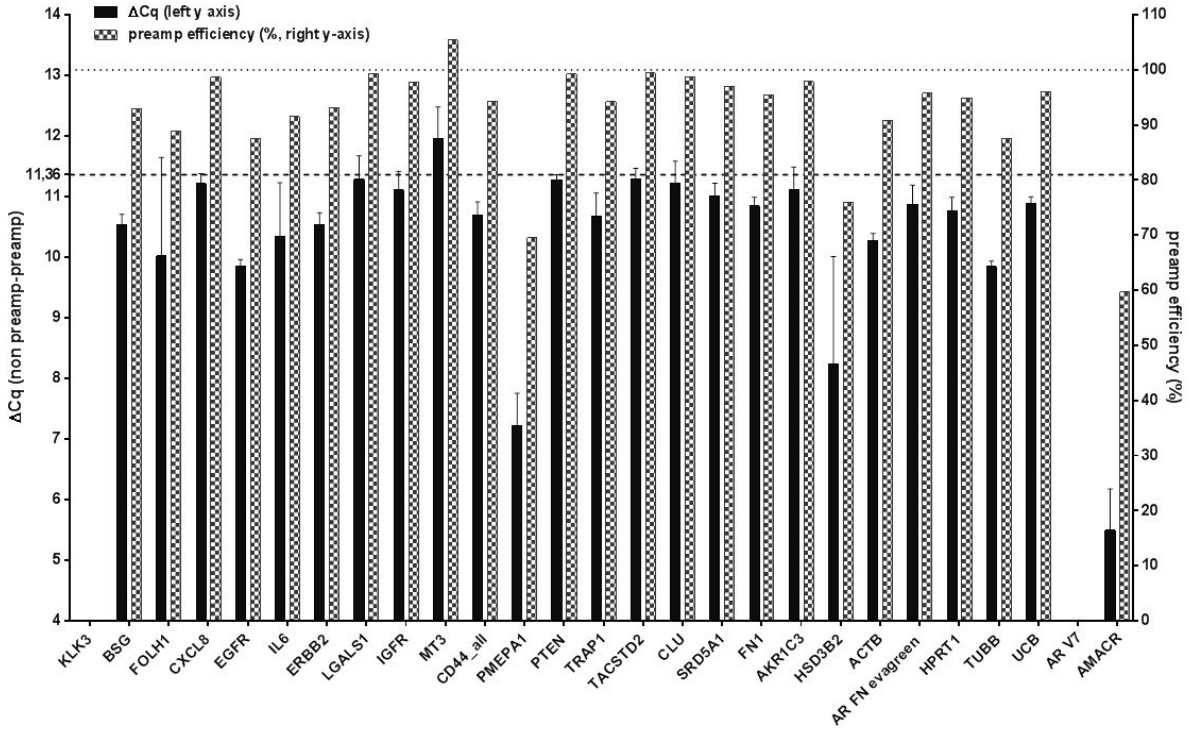


Figure 20: Preamplification efficacy (hatched) calculated from the difference between the Cq obtained after the preamplification (black) and the expected value of Cq (11.36).

A preamplification run with non-template control (NTC) showed some non-specific signals in the LGALS1, TRAP1 and AR genes (Figure 21). Nevertheless, the signals were usually generated after the 30th cycle and the efficacies of the genes were unaffected (Figure 20). CXCL8's non-specificity was random and negligible. All other genes proved to be highly specific even during the preamplification of all 27 genes at once.

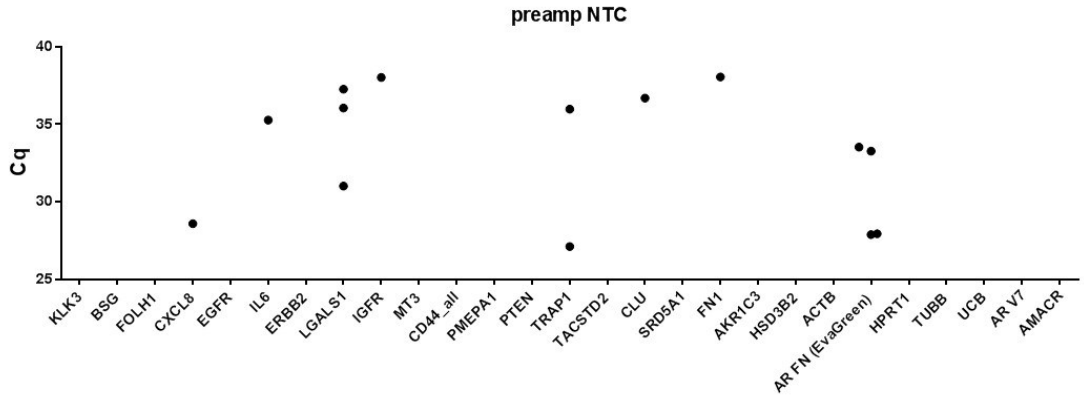


Figure 21: Results of preamplification done with 27 primer-probe sets by using the non-template control (NTC) as a sample.

In conclusion, the preamplification using primer-probes sets from the newly established gene expression panel achieved multiplication of the targeted genes without significantly changing their proportional representation in the sample. Such preamplification is thus suitable for use in a quantitative gene expression assay. Non-specific interactions detected in some primer-probe sets, i.e. LGALS1, TRAP1 and AR-FN, were weak and did not affect preamplification efficacy. All of the tested primer-probe sets were used in the final analysis; however, the results obtained during gene panel validation were taken into account during result evaluation (see Appendix 8.5) (Škereňová et al., submitted 2017).

4.3.3 Gene expression measurement on the BioMark platform

The results of the on-a-chip analysis are presented on a heat map in Figure 22.

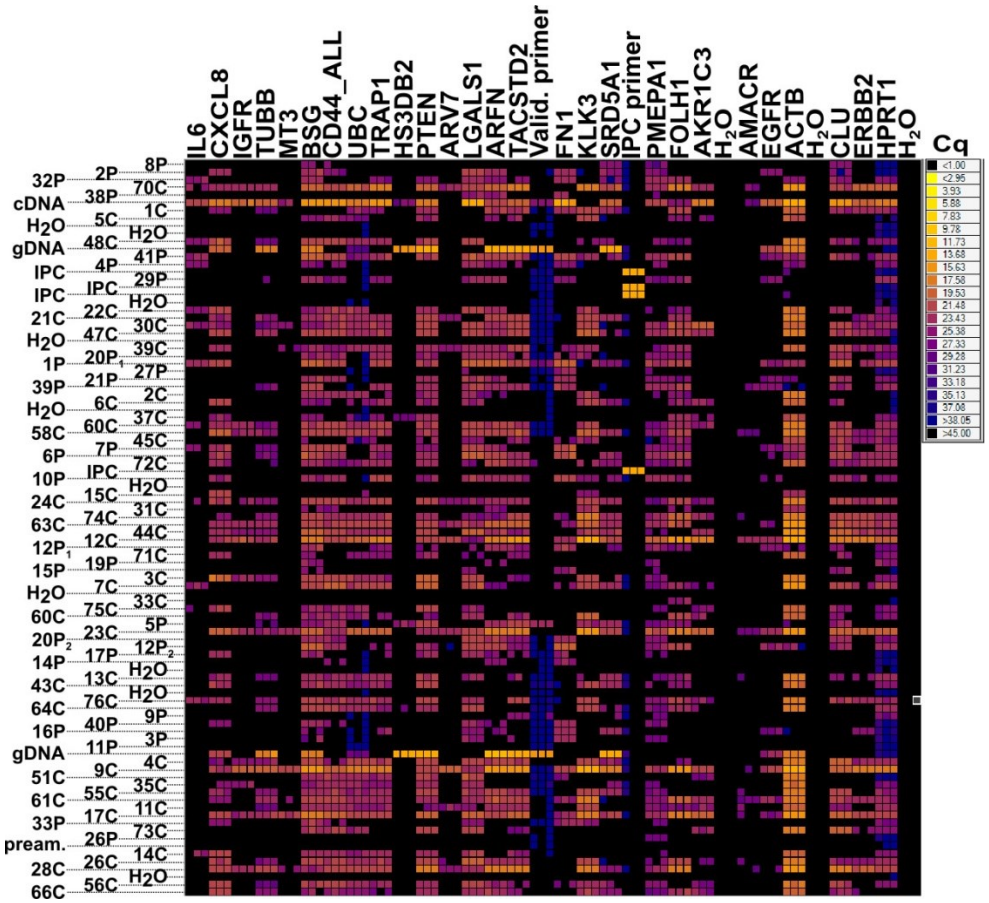


Figure 22: Results of the gene expression assay on the BioMark 96.96 Dynamic Array™ (Fluidigm, USA) chip in the form of a heat map based on the Cq of monitored genes.

The relative gene expression of CTC-enriched samples was calculated by the normalization of gene expression to the gene expression of actin, which was evaluated as an adequate reference gene for its consistent expression. The low detection rate of reference genes in the samples from the FFPE samples of the primary tumor may be caused by the presence of PCR inhibitors. Primary tumor expression data were thus analyzed on a zero/one scale, i.e. not expressed/expressed, instead of using the relative gene expression.

Cq cut-off for relevant gene expression was set to 30 for all samples, but 85% of generated signals were under Cq 26. The cycle number is slightly high because the specific characters of both types of samples were gained from a limited quantity of cells. Moreover, the mRNA from the primary tumor samples was affected by formalin fixation and long storage (von Ahlfen et al., 2007).

The UBC and HS3DB2 genes were removed from the final analysis because of a very low frequency of expression (Figure 22). Despite the weak preamplification efficacy of HS3DB2 gene primer-probe set, it was put to the final assay to maintain the conditions of the

pramplification validation. A primary tumor sample from the patient eleven (11P) was removed from the analysis because no signal under the established Cq was detected. CTC-enriched samples 15C, 31C and 33C were removed for sporadic and weak signals of expression.

To conclude, the final gene expression analysis comprised 25 genes (24 for the relative gene expression) and samples from 31 CRPC patients. Results from the primary tumor samples were evaluated on the 0/1 scale. Relative gene expression normalized to actin was determined from CTC-enriched samples. CTC-enriched samples proved to be a valid material for the gene expression analysis (see Appendix 8.5) (Škereňová et al., submitted 2017).

4.3.4 Analysis of monitored gene expression in circulating tumor cell-enriched and primary tumor samples

Frequency of gene expression of monitored genes differed significantly between CTC-enriched and primary tumor samples according to principal component analysis (Figure 23).

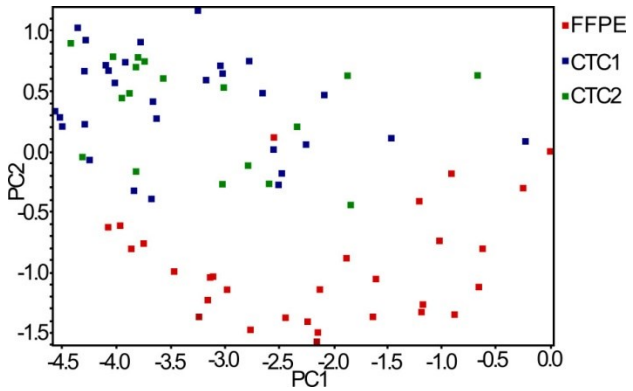


Figure 23: Principal component analysis (PCA) of patient samples according to the frequency of gene expression of genes monitored by the CTC-CRPC panel. CTC1-circulating tumor cell enriched samples at the time of diagnosis, CTC2-circulating tumor cell enriched samples during the therapy, FFPE-formalin-fixed paraffin-embedded

The difference is displayed also in an individual gene detection frequency between the two types of samples (Figure 24). Whilst the frequency of AR detection was similar in the primary tumor (FFPE) and CRPC (CTC1,2), its splice variant 7 (AR-V7) was found only in the samples from the advanced stage of the disease (CTC1,2). This is in concordance with current findings that the aberrant pathways of AR signaling reactivation, including AR-V7 pathway, appear later in PC, that is, during the development of castration resistance (Kong et al., 2015;

Watson et al., 2010; Zhang et al., 2011). The appearance of AR-V7 in CRPC patients may also be an effect of ADT used as a treatment prior CRPC diagnosis (Nakazawa et al., 2015).
Similarly, ERBB2 was found more often in CTC-enriched samples than in those from the primary tumor. ERBB2 is able to reactivate AR signaling by a ligand-independent mechanism and the expression of ERBB2 is thus related to CRPC development (Day et al., 2017; Gao et al., 2016). ERBB2 is also one of the antigens used for the immunomagnetic enrichment of CTCs by the AdnaTest. For this reason, a high capture of ERBB2 positive cells by this method is expected (Škereňová et al., 2016).

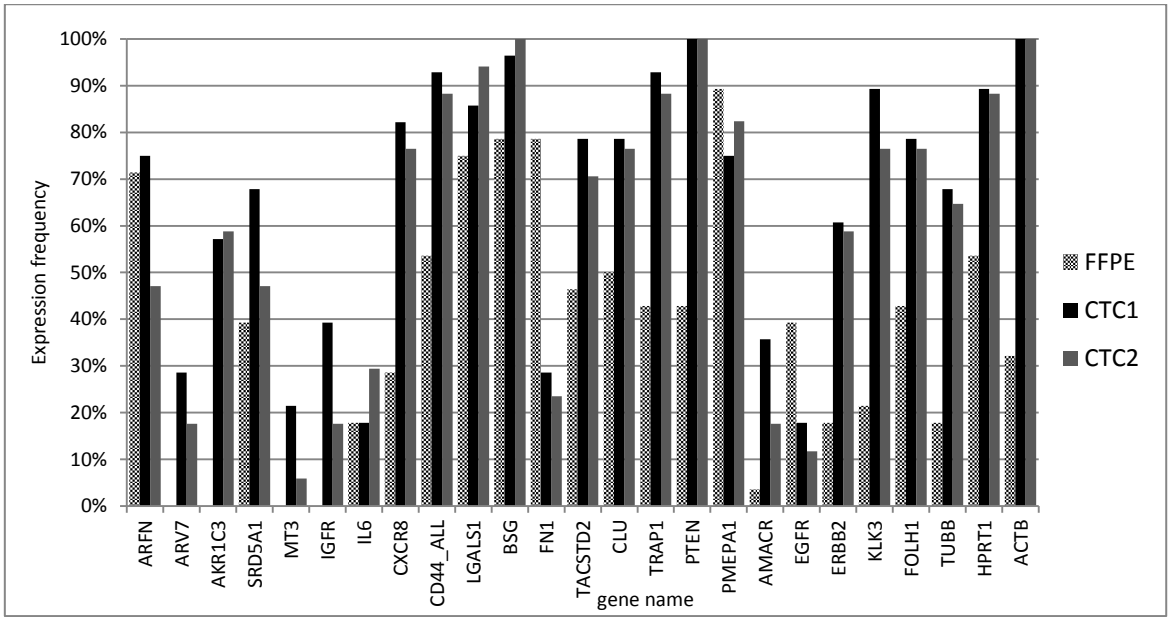


Figure 24: Frequency of individual gene expression in the primary tumor samples (FFPE), CTC-enriched fraction taken before docetaxel therapy (CTC1) and during the therapy (CTC2).

The expression of AKR1C3, MT3 and IGFR was found only in the CTC-enriched and not in the primary tumor samples. The expression of AKR1C3 had already been found to be associated with the androgen-independent status of PC. Stanbrough et al. showed its overexpression in metastases and androgen-independent tumors in contrast with the complete lack or weak expression of this gene in androgen-dependent primary tumors. AKR1C3 is a key enzyme of steroidogenesis in CRPC and plays a role in alternative mechanisms of AR signaling reactivation by synthesizing testosterone from alternative substrates (Adeniji et al., 2013; Stanbrough et al., 2006).
Similarly to our study, IGFR expression in CTC-enriched samples has been found in the study of breast cancer patients. Its expression has been associated with the suppression of the

anoikis enabling CTCs to survive in the bloodstream (Hensler et al., 2016; Werner and Sarfstein, 2014). IGFR, like MT3, interacts with the PI3K/AKT pathway, which is often upregulated in invasive cancer types. The expression of IGFR in CTC-enriched samples negatively correlated with the expression of PI3K/AKT inhibitor PTEN (CC=-0.49, p=0.002) and the expression of IGFR and MT3 weakly but significantly correlated with AR expression (CC_{IGFR}=0.31, p=0.042; CC_{MT3}=0.35, p=0.021) suggesting a possible relationship between these genes and the AR signaling pathway (Juang et al., 2013).

On the other hand, the expression of FN1 and EGFR was more frequently found in primary tumor samples. Both genes are negative prognostic markers and have already been connected with a more invasive disease phenotype, EMT and androgen-independent PC. The higher frequency of FN1 detection in the primary tumor samples may come from mesenchymal tissue which may be found in the biopsy samples. However, the prognostic role of FN1 expression in the CTC-enriched samples has already been published (Baron et al., 2006; Das et al., 2016). In our study, the expression of FN1 at the time of diagnosis was associated with a worse two-year DSS. A higher frequency of EGFR expression in the primary tumor samples could be caused by the lower efficacy of immunomagnetic enrichment of EGFR positive CTCs. The process of EMT in CTCs induced by EGFR signaling may result in the loss of epithelial markers, such as EpCAM and ERBB2, which are used for the immunomagnetic enrichment of CTCs by the AdnaTest. This could also explain the lower frequency of EGFR positive CTCs detected by the AdnaTest (Table 11). However, a similar study using the immunomagnetic detection of CTCs found EGFR in 25% of CTCs from CRPC patients, in comparison with 17% found in our study (Okegawa et al., 2016; Voon et al., 2013).

In conclusion, the genes involved in castration-resistance development and the alternative reactivation of AR signaling, i.e. AR-V7, ERBB2 and AKR1C3, were found more often in the samples enriched for CTCs than in those of primary tumors. If possible, the primary tumor tissue samples should be stored in a solution dedicated to preserving mRNA e.g. RNAlater (Sigma-Aldrich) instead of using FFPE samples. The CTC-enriched samples stored in the freezer after the AdnaTest analysis, can be use in the gene expression analysis. They can be recommended as a valid material for gene expression assays (see Appendix 8.5) (Škereňová et al., submitted 2017).

4.3.5 Characterization of relative gene expression in circulating tumor cell enriched samples

A relative gene expression of the CTC-enriched samples was calculated, compared with the results of the AdnaTest and associated with patient survival and therapy response.

4.3.5.1 Analysis of monitored gene expression

Gene expression between all CTC-enriched samples is comparable because all of them were prepared from the same volume of blood. However, the total number of CTCs may differ between samples. In our analysis, a relative gene expression was adjusted to the background of each sample, the gene expression of actin which is expressed in both leukocytes and CTCs (Figure 25).

The relative expression was also determined in one sample evaluated by the AdnaTest as CTC negative (patient 39 during therapy) and in one sample evaluated as CTC negative but with AR expression (patient 41 at the time of CRPC diagnosis). The analysis of genes expressed in the CTC negative samples, i.e. AKR1C3, IL6, CXCL8, CD44, LGALS1, BSG, TACSTD2, CLU, PTEN, PMEPA1, ERBB2 and HPRT1 showed that all of these genes may be expressed by leukocytes (The Scripps Research Institute, 2017). Despite the fact that these genes have a close relation to PC development and most of them were found to be overexpressed in CRPC (Table 6), the signal may also originate from the leukocytes remaining in the CTC-enriched samples after the immunomagnetic separation step (Allan and Keeney, 2010; Sieuwerts et al., 2009). Similar results have recently been published by a research group studying CTCs in breast cancer patients (Hensler et al., 2016).

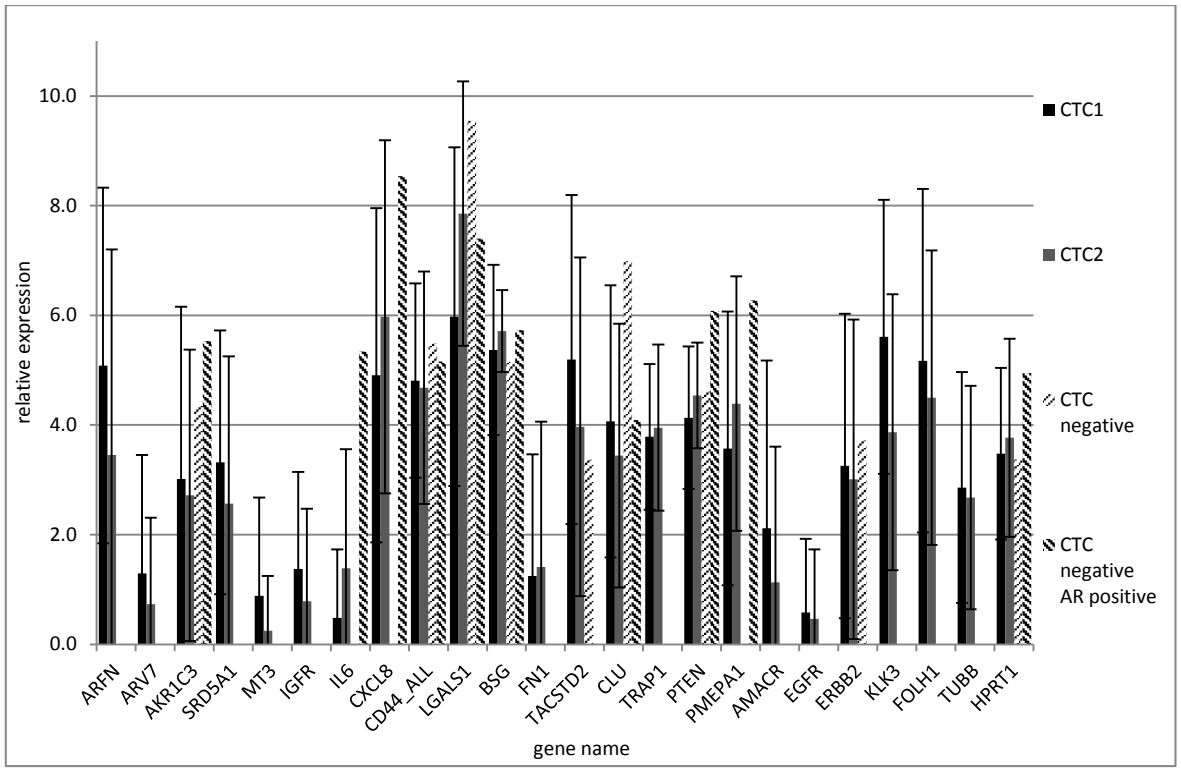


Figure 25: Relative gene expression of monitored genes in CTC- enriched samples before therapy (CTC1, N=27), during therapy (CTC2, N=16), in one sample evaluated by the AdnaTest as CTC negative (hatched, N=1), in one sample evaluated as CTC negative but with AR expression detected by the AdnaTest (hatched, N=1).

The real ratio of CTCs and leukocytes in the individual samples is unknown. It is assumed that the quantity of leukocytes after immunomagnetic enrichment is in thousands of cells per sample and the concentration of CTCs in the blood of cancer patients varies from one to thousands per milliliter (Table 3) (Allan and Keeney, 2010). Moreover, CTCs which do not express any of the genes monitored by the AdnaTest may theoretically be present in samples evaluated as CTC negative. For these reasons, the origin of the signal of the genes detected in CTC negative patients is hard to assess. The connection of some of these genes with patient survival and therapy response suggests some role of these genes in CRPC, regardless of their origin.

Patients who experienced ≥30% decrease in sPSA had increased expression of PTEN. Two patients whose PTEN expression decreased had a worse sPSA outcome (Figure 26). The PTEN expression was also associated with a better two-year DSS in both measurements (p_{CTC1}=0.026, p_{CTC2}<0.001).

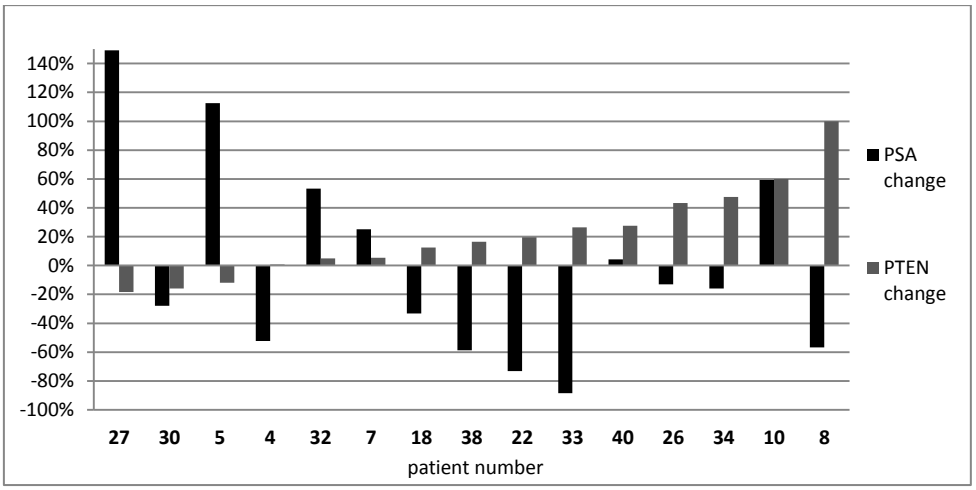


Figure 26: Patients ordered from the lowest to the highest change in PTEN expression with the corresponding relative change in PSA serum level.

The better two-year DSS was also associated with the expression of SRD5A1 (p=0.038) and CD44 (p=0.019) during therapy. In contrast, the expression of FN1 (p=0.035) and ERBB2 (p=0.047) at the time of CRPC diagnosis represented a negative predictive marker for two-year DSS. CLU expression was associated with a worse sPSA response.

To conclude, genes from the established gene panel which were found in CTC negative samples were in the subsequent analysis considered as a potential contamination; not reflecting the actual expression of PC CTCs. However, statistical analysis found some interesting connections between these genes and patient prognosis. A larger study, comprised of more CTC negative samples, is required to explore which percentage of the expression of these genes comes from the background and which comes from CTCs. Despite highly specific immunomagnetic enrichment, the CTC-enriched samples gained from the AdnaTest have to be handled as a mixture of cDNA from CTCs and leukocytes (see Appendix 8.5) (Škereňová et al., submitted 2017).

4.3.5.2 Correlation between AdnaTest results and gene expression measured on the BioMark platform

The concentration of PCR fragments determined by the AdnaTest showed very good correlation with the relative gene expression of corresponding genes determined on the BioMark platform (Table 13). The worse correlation of EGFR is probably caused by the rare detection of this gene in both assays (Škereňová et al., submitted 2017). The positive effect of normalization to actin was not confirmed as associated with the gene expression (Škereňová

et al., 2016). The influence of the normalization was inconsistent, which was probably caused by the shape of the PCR amplification curve, which results in a non-proportional multiplication of the fragments in the late, i.e. plateau, phase of the reaction.

Table 13: Correlation between relative gene expressions measured on the BioMark platform and PCR fragment concentrations before and after normalization to actin measured by the AdnaTest.

Monitored PCR fragment and gene	Assay name of monitored PCR fragment /gene	Before normalization (N=44)	After normalization (N=44)
Epidermal growth factor receptor	**EGFR/EGFR**	CC=0.387, p=0.009	CC=0.320, p=0.034
Prostate specific antigen	**PSA/KLK3**	CC=0.704, p<0.001	CC=0.796, p=<0.001
Prostate specific membrane antigen	**PSMA/FOLH1**	CC=0.715, p<0.001	CC=0.688, p<0.001
Androgen receptor*	**AR/ARFN**	CC=0.774, p<0.001	-

* androgen receptor (AR) was not normalized to actin because of the single-plex character of AR detection by the AdnaTest. CC=correlation coefficient

To summarize, the PCR fragment concentrations monitored by the AdnaTest reflect the expression of corresponding genes and represent a form of semi-quantitative gene expression analysis.

4.3.5.3 Changes in gene expression during therapy

A principal component analysis was performed to identify which genes' relative expression changed the most during the therapy (Figure 27). The identified genes, i.e. FOLH1, AR, KLK3, TACSTD2 and ERBB2, present the response of CTCs to docetaxel therapy. The trend in these genes' expression is depicted in a chart comparing the change in the relative expression in 16 patients who had gene expression results from both CTC measurements (Figure 28).

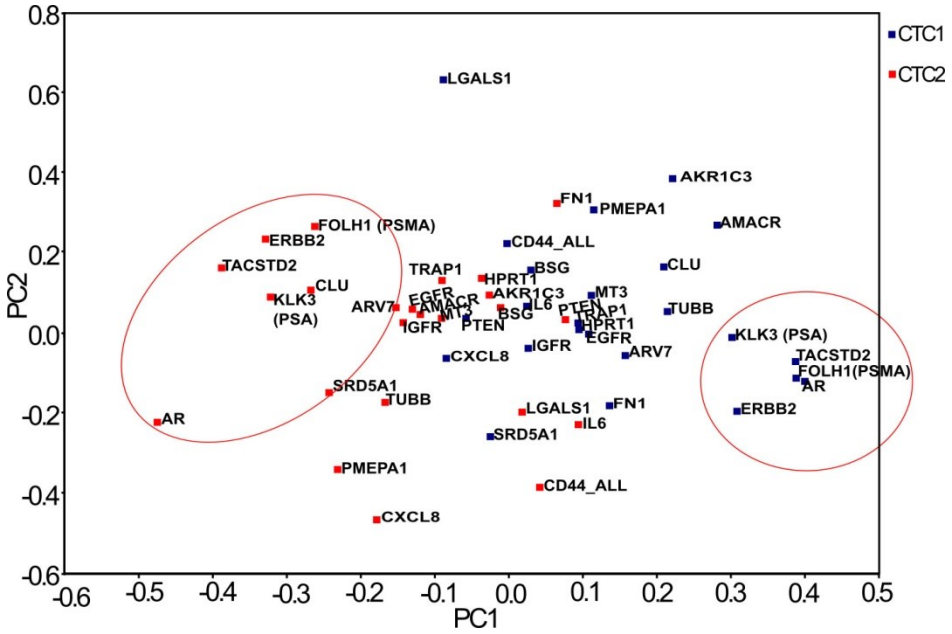


Figure 27: Comparison of monitored gene expression between CTC-enriched samples taken before (CTC1) and during therapy (CTC2) by principal component analysis (PCA). Genes with the most different expression between the groups are circled.

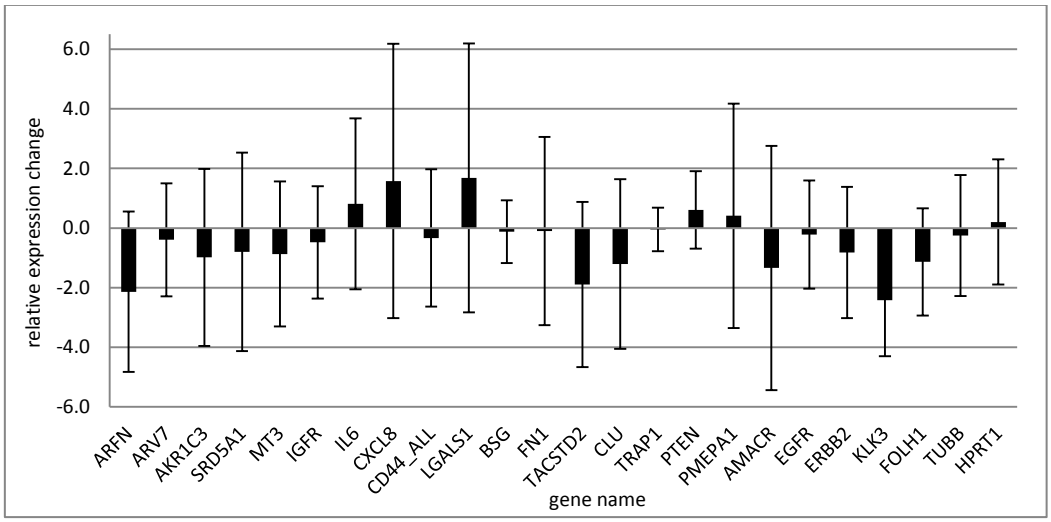


Figure 28: Change in relative gene expression of monitored genes in 16 patients between CRPC diagnosis and after the 3rd cycle of docetaxel therapy.

The role of the androgen signaling pathway in PC has already been discussed in Chapter 4.2.1 in the context of the AdnaTest results. Similar results were obtained from the gene expression analysis. The major change in expression during docetaxel therapy was observed in genes involved in the AR signaling pathway, i.e. AR, KLK3 and FOLH1.

The expression of KLK3 was measured in the majority of CTC-enriched samples - in 90% at the time of CRPC diagnosis and 80% during therapy (Figure 24). Relative gene expression of

KLK3 significantly decreased during therapy in all patients when CTC1 and CTC2 groups were compared (Figure 28). The frequency of KLK3 expression as well as its decrease during therapy corresponds very well with the findings published by Dijkstra who found KLK3 in 90% of CRPC patients and observed a decrease in expression during docetaxel therapy. In contrast with Dijkstra, a correlation between KLK3 expression in CTCs and the sPSA level was not observed in our study, supporting notion that CTCs are not the main driver of the sPSA level in CRPC (Dijkstra et al., 2014; Yao et al., 2014). Similarly, a decrease in the relative expression of FOLH1 observed during the therapy may be a sign of therapy efficacy, because FOLH1 is considered to be a marker of PC progression.

The AR signaling pathway may also be activated by the ERBB2. The decrease of ERBB2 expression, which is overexpressed during disease progression, may be a sign of a positive response to the therapy (Gao et al., 2016). Similarly, the decrease in TACSTD2 expression which is connected with the taxane resistance of PC cells during the treatment may be also count towards the positive effect of docetaxel therapy (Shvartsur and Bonavida, 2015).

Whilst the relative gene expression of genes monitored in our study is related to actin, which comes from both CTCs and leukocytes present in the samples' background, the decrease in AR-related gene expression may result from the overall decrease in CTCs number during docetaxel therapy (Bitting et al., 2015; Olmos et al., 2009). However, expression did not decrease in all prostate-specific genes and each patient exhibited unique characteristic of gene expression development during the treatment. The decreased relative gene expression is thus probably caused by both, the decreased in CTC number and gene expression change during the therapy.

In conclusion, the response to the docetaxel therapy was characterized by a decrease in the expression of genes associated with the AR signaling pathway, i.e. KLK3, FOLH1 and AR, in the CTC-enriched samples. Since the expression of ERBB2 and TACSTD2 was present in the CTC negative samples, these results must be evaluated with caution. The individual molecular characteristics of each tumor as well as a possible decrease in CTCs, are probably involved in gene expression changes during the therapy (see Appendix 8.5) (Škereňová et al., submitted 2017).

4.3.5.4 Clustering of samples according to gene expression

To examine patient samples according to their gene expression, a cluster analysis was performed. It divided the CTC-enriched samples into two clusters. The expression pattern was

represented by three main groups of genes. The major difference between the clusters is represented by the gene group consisting of KLK3, FOLH1, AKR1C3, TACSTD2 and AR (Figure 29). The clusters were termed “low” or “high” according to their predominant expression pattern.

Despite the previous results (Figure 27), the cluster analysis put the samples from one patient mostly into the same cluster regardless of whether they were taken before or in during the therapy. From sixteen monitored patients all except three ended up in the same cluster. Three patients who changed cluster during the therapy (circled in Figure 29) were all “high” at the time of CRPC diagnosis and became “low” during docetaxel therapy. The previously shown decrease in AR-related genes during therapy was thus verified. However, its influence on the overall gene expression was weaker than the individual gene expression pattern of each patient.

It is known that CTC expression and quantity differs both within and between patients (Bitting et al., 2015; de Bono et al., 2008; Chen et al., 2013; Punnoose et al., 2015; Reyes et al., 2014). Our results suggest that intra-patient variance is smaller than inter-patient variance during the first cycles of docetaxel therapy. The “high” and “low” expression groups were compared on the basis of best response, sPSA change, OS and DSS but no statistically significant differences were found.

Genes with a major impact on sample clustering correspond very well with the genes whose expression changed during the therapy (Figure 27). In the clustering analysis the ERBB2 gene was replaced by the AKR1C3 gene. Both of these genes were also expressed in CTC negative samples and can thus be the result of leukocyte contamination in the samples (Figure 25). On the other hand, AKR1C3 is the key enzyme of steroidogenesis and has thus a clear connection to the AR signaling pathway, as already discussed in Chapter 4.3.4 (Adeniji et al., 2013).

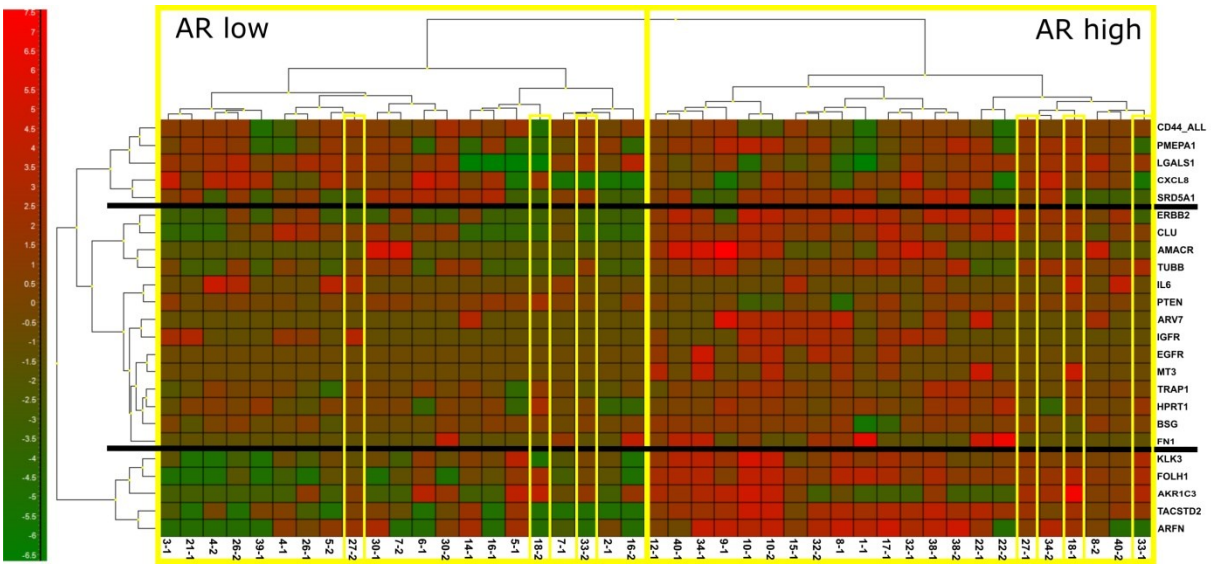


Figure 29: Clustering of CTC-enriched samples according to their expression pattern of monitored genes (yellow boxes). Clustering of monitored genes according to their co-expression in the samples (black lines). Samples from the patients which changed groups during the therapy are marked by yellow rectangles. 1-samples taken at the time of CRPC diagnosis; 2-samples taken during docetaxel therapy

In conclusion, CTC-enriched samples obtained from CRPC patients can be divided according to their low or high expression of monitored genes. This status generally remained consistent during the first three cycles of docetaxel therapy. Docetaxel therapy may result in the downregulation of gene expression in some patients. Gene expression in CTC-enriched samples differs more between individual patients in the tested cohort than within the samples from one patient collected at various therapy time points. The existence of this personal pattern could stress the ability of this method to individually characterize the molecular profile of the disease (see Appendix 8.5) (Škereňová et al., submitted 2017).

4.3.5.5 Role of androgen receptor and its splice variant 7

In accordance with the current literature, our study proved that AR plays a crucial role in CTCs from CRPC patients (Stanbrough et al., 2006; Waltering et al., 2012; Wyatt and Gleave, 2015). The detailed analysis of AR and AR-V7 are presented in Appendix 8.5 (Škereňová et al., submitted 2017).

The expression of AR determined by gene expression analysis on the BioMark platform correlated very well with the AR expression determined by the AdnaTest (Table 13). The differences in seven (out of 44) samples evaluated as AR positive by the AdnaTest and as AR

negative by the gene expression analysis may be caused by the use of different primers in each method (Figure 30).

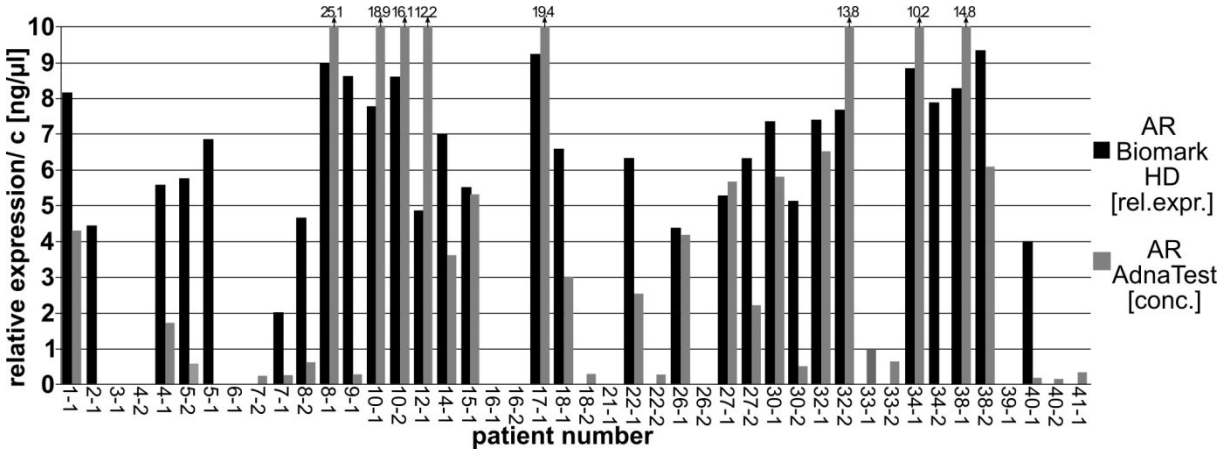


Figure 30: Comparison of the relative gene expression of AR determined by analysis on the BioMark platform and the final concentration of AR PCR products determined by the AdnaTest.

Similar to the AR PCR fragment concentration discussed in the chapter 4.2.2., the relative expression of AR as a result of the gene expression analysis was associated with worse DSS and a worse sPSA therapy response. Patients with AR expression at the time of CRPC diagnosis had a shorter DSS (Figure 31A). The expression of AR correlated with patients' sPSA level at the time of CRPC diagnosis (CC=0.79164, $p<0.001$) and during docetaxel therapy (CC=0.5231, $p=0.0376$). Patients with AR expression had a significantly higher relative change in sPSA during the therapy, suggesting a worse therapy response (Figure 31B).

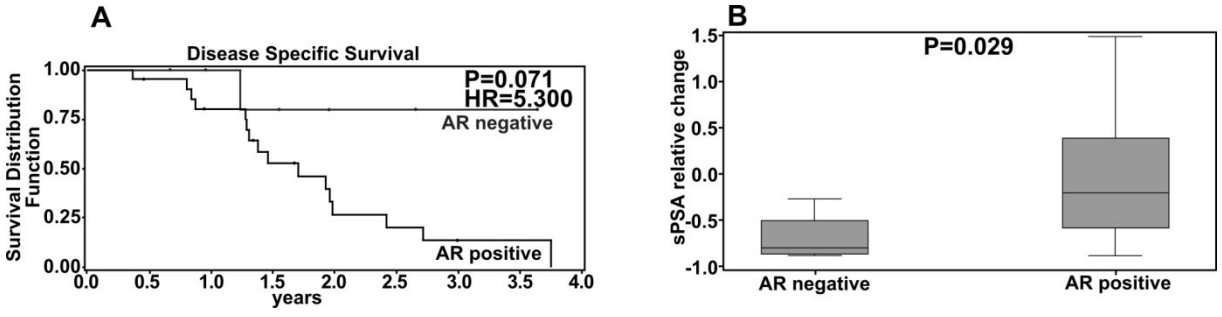


Figure 31: Prognostic value of AR expression at the time of CRPC diagnosis: A) Kaplan-Meier analysis of the DSS of AR positive and AR negative patients B) relative change of sPSA in AR positive and AR negative patients.

As was noted above, the constitutively active AR-V7 represents one of the known mechanisms of the aberrant reactivation of the AR signaling pathway in PC. In concordance with a theory about CRPC development (Waltering et al., 2012), we found AR-V7 only in CTC-enriched samples and not in any sample of the primary tumor (N=31). The expression of AR-V7 was detected in 9 out of 28 patients followed. Eight patients (30%) was AR-V7 positive at the time of CRPC diagnosis and 3 patients (19%) during docetaxel therapy. From five patients who underwent both measurements two remained AR-V7 positive, two became AR-V7 negative and one became AR-V7 positive during docetaxel therapy. AR-V7 was always expressed together with the full-length AR. AR-V7 was found in 38% of AR positive samples and the percentage was constant at the time of CRPC diagnosis and during therapy (Figure 32).

According to current knowledge, the AR-V7 may be present only in a subpopulation of CTCs in AR-V7 positive patients. However, this subpopulation of cancer cells can multiply when antiandrogen-targeted therapy is applied causing a relapse of the disease and therapy inefficacy (Jiang et al., 2010). The detection of AR-V7 thus represents key information to CRPC therapeutic decision-making.

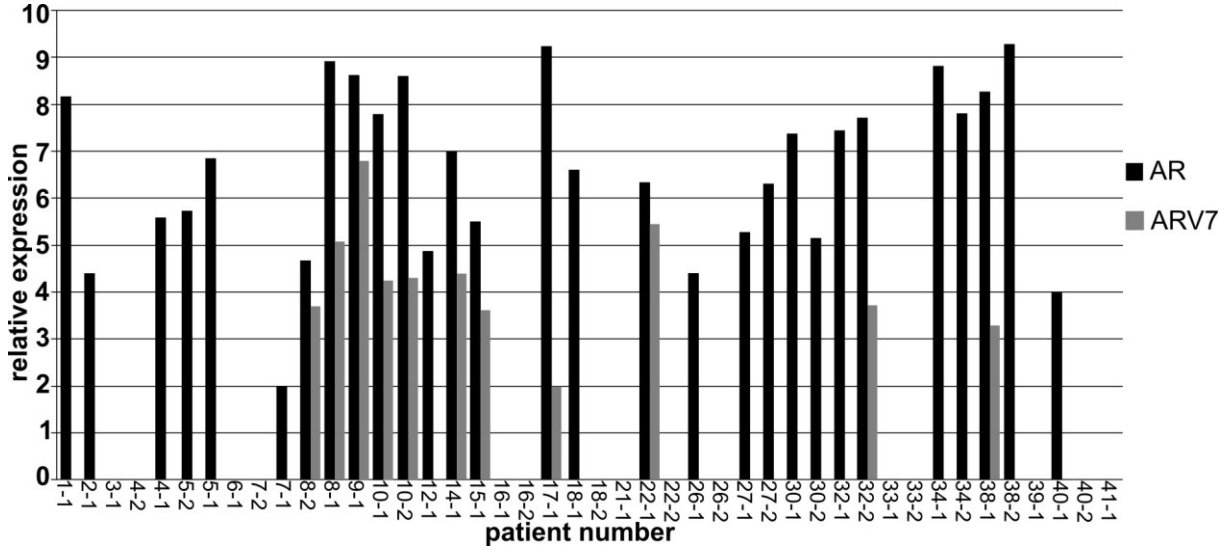


Figure 32: Relative expression of AR and AR-V7 in CRPC patients. 1-at the time of CRPC diagnosis; 2-during docetaxel therapy.

The AR-V7 splice variant is studied especially because of its role in the development of anti-androgen therapy resistance. Taxane therapy, e.g.docetaxel, was suggested as a tool to reverse an antiandrogen therapy resistance in CRPC patients caused by AR-V7 expression and renew their sensitivity to antiandrogen therapy (Nakazawa et al., 2015; Onstenk et al., 2015;

Sprenger et al., 2015). In concordance with the Nakazawa's theory, the changes in AR-V7 expression during the docetaxel therapy, observed in our study, were sometimes favorable. However, patient 32 became AR-V7 positive and patients 8 and 10 stayed AR-V7 positive during the therapy. Longer docetaxel therapy may thus be required to fully affect anti-androgen resistance in CRPC patients (Figure 11).

To conclude, AR status can be determined by both the AdnaTest and the BioMark. In our study, AR status determined by the AdnaTest showed a stronger connection with patient survival. The relative expression of AR and AR-V7 can be determined in CTC-enriched samples. Their detection may have a prognostic value for CRPC patients and correlates with sPSA therapy response. The determination of these markers can be of particular use in therapeutic decision-making concerning new antiandrogens (see Appendix 8.5) (Škereňová et al., submitted 2017).

5 Conclusions

Starting from method implementation and characterization through clinical testing for patient prognosis and therapy-response evaluation, followed by the use of obtained samples for high-throughput gene expression analysis, it has been shown that the AdnaTest method can serve not only for CTC detection but also as a molecular characterization technique in CRPC. This method is useful in patient prognosis determination and in therapeutic decision-making.

- **To implement the AdnaTest method and to evaluate its characteristics on patient samples.**

The principle and the semi-quantitative character of the AdnaTest method were verified by using a PC cell line (LNCaP). The characteristics of the method determined on patient samples were described and compared with manufacturer information and the current literature.

- **To correlate the results of CTC detection by the AdnaTest to the clinico-pathological characteristics of CRPC patients.**

CTCs were found in 85% of CRPC patients at the time of diagnosis and in 45% of the patients after the 3rd cycle of docetaxel therapy. A positive CTC test was associated with worse survival and a higher sPSA level. CTCs detected by the AdnaTest were associated with an ongoing metastatic process in advanced cancer patients. The monitoring of EGFR and AR status by the AdnaTest was associated with DSS.

- **To design and test a new multi-marker gene expression panel to monitor CTC character during CRPC therapy.**

A new gene expression panel for liquid biopsy in CTC testing during CRPC was designed and tested. The high-throughput qPCR analysis on the BioMark platform was successfully preformed, gene expression results for 25 out of 27 genes in 75 out of 79 (48 CTC-enriched and 31 FFPE primary tumor) samples from 31 CRPC patients were obtained. The quality of FFPE primary tumor samples was insufficient for relative gene expression determination, but the CTC-enriched samples proved to be a valid material for the analysis of the gene panel expression.

- **To explore the use of CTC-enriched samples in the high-throughput qPCR analysis.**

The expression of genes in the designed gene expression panel was successfully measured and relatively-quantified in the CTC-enriched samples on the BioMark platform. Genes

involved in castration-resistance development and alternative reactivation of the AR signaling pathway were more frequently found in CTC-enriched samples than primary tumor samples. Nevertheless, the CTC-enriched samples contain a background signal from leukocytes remaining in the samples after the immunomagnetic separation. The significance of the influence of this upon gene expression results requires further study. However, cancer-specific genes should not be influenced by this phenomenon.

- **To evaluate the semi-quantitative results of the AdnaTest by determining their correlation with the qPCR results measured on the BioMark platform.**

The semi-quantitative results of the AdnaTest correlated very well with the relative gene expression determined on the BioMark platform. The weaker correlation of EGFR is probably caused by its low frequency of detection. The AdnaTest may be, as long as the principle of the method is taken in account, evaluated as a semi-quantitative gene expression assay and consequently as a liquid biopsy method.

- **To investigate the gene expression in CTC-enriched samples and its relation to patient prognosis and therapy response with a special focus on the marker of anti-androgen therapy resistance, i.e. AR-V7.**

AR related genes play a crucial role in CTCs from CRPC patients. A different expression of AR-related genes divides CRPC patients into “low” and “high” expression clusters. Despite the observed decrease in AR-related genes during docetaxel therapy, the cluster classification does not change within the first three cycles of docetaxel therapy. The gene expression of CTC-enriched samples varies more between patients then between samples from one patient taken before and during therapy. The differences in gene expression within and between patients may result from the absolute quantity of CTCs in the samples and from the molecular characteristic of the disease.

AR expression can be semi-quantitatively determined by the AdnaTest. The quantitative measurement of AR expression and the presence of its splice variant AR-V7 can be determined from CTC-enriched samples by gene expression analysis. Their detection correlates with the sPSA response and survival of CRPC patients. The determination of these markers can be of a particular use in making therapeutic choices concerning new anti-androgens.

6 Publications:

6.1 Papers

- **Cirkulující nádorové buňky a prognóza karcinomu prostaty.**

  Čapoun, O., Soukup, V., Mikulová, V., Jančíková, M., Honová, H., Kološtová, K., Zima, T., Hanuš, T.
  2014. Cas. Lek. Cesk. 153, 72–77.
  bez IF

- **Prognosis of Castration-resistant Prostate Cancer Patients - Use of the AdnaTest® System for Detection of Circulating Tumor Cells.**

  Čapoun, O., Mikulová, V., Jančíková, M., Honová, H., Kološtová, K., Sobotka, R., Michael, P., Zima, T., Hanuš, T., Soukup, V.
  2016. Anticancer Res. 36, 2019–26.
  IF 1.937

- **The characterization of four gene expression analysis in circulating tumor cells made by Multiplex-PCR from the AdnaTest kit on the lab-on-a-chip Agilent DNA 1000 platform.**

  Škereňová, M., Mikulová, V., Čapoun, O., Zima, T.
  2016. Biochem. Medica 26, 103–113.
  IF 2.934

- **Circulating tumor cells and serum levels of MMP-2, MMP-9 and VEGF as markers of the metastatic process in patients with high risk of metastatic progression.**

  Skerenova, M., Mikulova, V., Capoun, O., Zima, T., Tesarova, P.
  2017. Biomed. Pap., e-pub 16.5.2017.
  IF 0.894

- **Gene expression analysis of immunomagnetically-enriched circulating tumor cell fraction in castration-resistant prostate cancer**

  Škereňová, M., Mikulová, V., Čapoun, O., Švec, D., Soukup, V., Honová, H., Hanuš, T., Zima, T.
  submitted to Clinical Genitourinary Cancer in August 2017
  IF 2.535

6.2 Posters and lectures

- **Student scientific conference, 1st Faculty of Medicine, Charles University in Prague, Czech Republic, May 2012**

  Jančíková, M.
  Presentation: Detekce a charakterizace cirkulujících nádorových buněk u pacientů s kastračně rezistentním karcinomem prostaty

- **XXIII. Biochemický sjezd, Olomouc, Czech Republic, September 2012**

  Jančíková, M., Zima, T., Mikulová, V. Soukup, V., Čapoun, O., Honová, H., Hanuš, T., Šírová, M.
  Poster: Detekce a charakterizace cirkulujících nádorových buněk u pacientů s kastračně rezistentním karcinomem prostaty

- **International Society of Oncology and Biomarkers (ISOBM), Jerusalem, Israel, October 2012**
  Jančíková, M., Zima, T., Mikulová, V., Čapoun O., Soukup, V., Honová, H.
  Poster: Circulating tumor cells as a biomarker for the patients with castration resistant prostate cancer

- **PragueOnco, Prague, Czech Republic, January 2013**
  Jančíková, M., Mikulová, V., Soukup, V., Čapoun, O., Hanuš, T., Honová, H., Zima, T.
  Poster: Nové možnosti monitorování pacientů s kastračně rezistentním karcinomem prostaty pomocí cirkulujících nádorových buněk

- **World CTC meeting, Berlin, Germany, April 2013**
  Jancikova, M., Mikulova, V., Capoun, O., Soukup, V., Honova, H., Sirova, M., Zima, T.
  Poster: Circulating tumor cells as a source of information about castration-resistant prostate cancer

- **Symposium on Minimal Residual Cancer (ISMRC), Paris, France, September 2013**
  Jancikova, M., Mikulova, V., Capoun, O., Soukup, V., Honova, H., Sirova, M., Zima, T.
  Poster: Circulation tumor cells in castration resistant prostate cancer

- **2nd International Symposium ACTC: Advances in Circulating Tumor Cells, Rethymnon, Greece, October 2014**
  Jancikova, M., Mikulova, V., Capoun, O., Soukup, V., Tesarova, P., Honova, H., Zima, T.
  Poster: Correlation between the levels of metastases-related serum markers and the presence of circulating tumor cells in breast and prostate cancer patients

- **Student scientific conference, 1st Faculty of Medicine, Charles University in Prague, Czech Republic, May 2015**
  Jancikova, M., Mikulova, V.
  Presentation: Stanovení sérových metastatických markerů a cirkulujících nádorových buněk u pacientek s pokročilým HER2 pozitivním karcinomem prsu

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8 Appendix

8.1 Cirkulující nádorové buňky a prognóza karcinomu prostaty.

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8.5 Gene expression analysis of immunomagnetically-enriched circulating tumor cell fraction in castration-resistant prostate cancer

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