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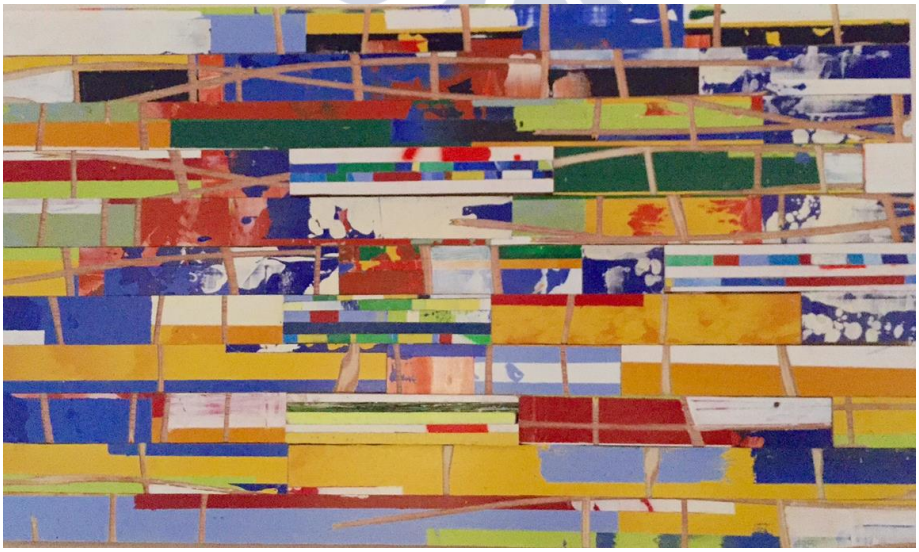
Clinical application of circulating tumor cells in patients with metastatic castration-resistant prostate cancer



PhD Tesis, 2015

Luis León Mateos

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Clinical application of circulating tumor cells in patients with metastatic castration-resistant prostate cancer

Memoria que presenta:
Luis León Mateos
para optar ao grado de Doutor en Medicina

Fdo. Luis León Mateos
Santiago de Compostela, Novembro 2015

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El **Doctor Rafael López López** profesor asociado de ciencias de la salud, en el departamento de Medicina de la Universidad de Santiago de Compostela, Jefe de Servicio de Oncología Médica del Hospital Clínico Universitario de Santiago de Compostela, el **Doctor Ihab Abdulkader** profesor asociado de ciencias de la salud, en el departamento de Medicina de la Universidad de Santiago de Compostela, Adjunto en el Servicio de Anatomía Patológica del Hospital Clínico Universitario de Santiago de Compostela y la **Doctora Laura Muinelo Romay** investigadora del laboratorio de Oncología Médica traslacional del IDIS.

INFORMAN:

Que el presente trabajo titulado "Clinical application of circulating tumor cells in patients with metastatic castration-resistant prostate cancer" se ha realizado bajo su dirección por el licenciado en Medicina Luis León Mateos y se encuentra en condiciones de presentarse y defenderse como tesis doctoral ante el tribunal correspondiente en la Universidad de Santiago de Compostela.

Para que así conste, se expide el presente certificado en Santiago de Compostela a 12 de Noviembre de 2015.

Dr. Rafael López Dr. Ihab Abdulkader Dra. Laura Muinelo

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"The answer is blowing in the wind"

Robert Allen Zimmerman

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Por mis padres.

Para Javier, Sabela, Miguel, Pedro.

A Bea.

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ABSTRACT.

Background. Prostate cancer (PCa) is the most common diagnosed male malignancy in the Western world. One third of patients will develop metastatic castration resistant prostate cancer (mCRPC).

To monitor systemic disease, the study of circulating tumor cells (CTCs) can be a supplement or an alternative to the serum PSA and imaging methods. In the present study we assessed the value of CTC count and molecular characterization to manage mCRPC patients.

Methods. Blood samples from 29 mCRPC patients treated with first line taxanes were analyzed at four different time points. Samples were first processed by the Cellsearch platform. Besides we did a CTC isolation and a gene expresson analysis by RT-qPCR at baseline in 29 mCPRC. In addition a whole gene expression analysis was carried out on blood samples extracted from 9 patients and from 6 healthy donors.

Results. CTC count worked as a prognostic factor: median OS was 16 months for those patients with ≥5 CTCs at baseline versus not reached for those < 5 CTCs. In addition to the CTC count we identified a molecular CTCs-signature that could be useful for the initial diagnosis, prognosis and therapy monitoring. Thus, high levels of AR, CYP19 and GDF15 were associated with poor PFS rates while AR, GDF15 and BIRC5 were also found as consistent predictors of OS in the univariate analysis. Finally after the global gene expression approach we found a general stress-survival phenotype in the CTC population of mCRPC patients partially based on cell proliferation, apoptosis, adhesion and migration.

Conclusions. In our cohort CTC count using CellSearch technology has prognostic value. In parallel, we have also demonstrated the feasibility of an alternative method of CTC isolation and gene expression analysis, identifying a panel of genes with prognostic relationship and deserves to be explored for the treatment monitoring.

Keywords. Circulating tumor cells; CTC; castration resistant prostate cancer; global gene expression analysis.

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Chapter 1. General Introduction

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1. Prostate cancer overview.

1.1 Epidemiology.

Prostate cancer is the most prevalent male urogenital malignancy and the second leading cause of cancer death among men in industrialized countries, with 1.1 million new cases and 307,500 estimated deaths to have occurred in 2012. Age-standardized incidence and mortality per 100,000 inhabitants per year were 96.0 and 19.3 in Europe and 96.8 and 15.2 in Spain, respectively.

About two-thirds of these cases were diagnosed in economically developed countries, with the highest incidence in Northern and Western Europe, Northern America, Oceania, and some Caribbean island nations, and lowest in Asia. Much of the variation reflects differences in the use of prostate specific antigen (PSA) testing the aging population, the use of improved diagnostic techniques, programs for early detection and increased surgical procedures performed on benign prostate disease (1).

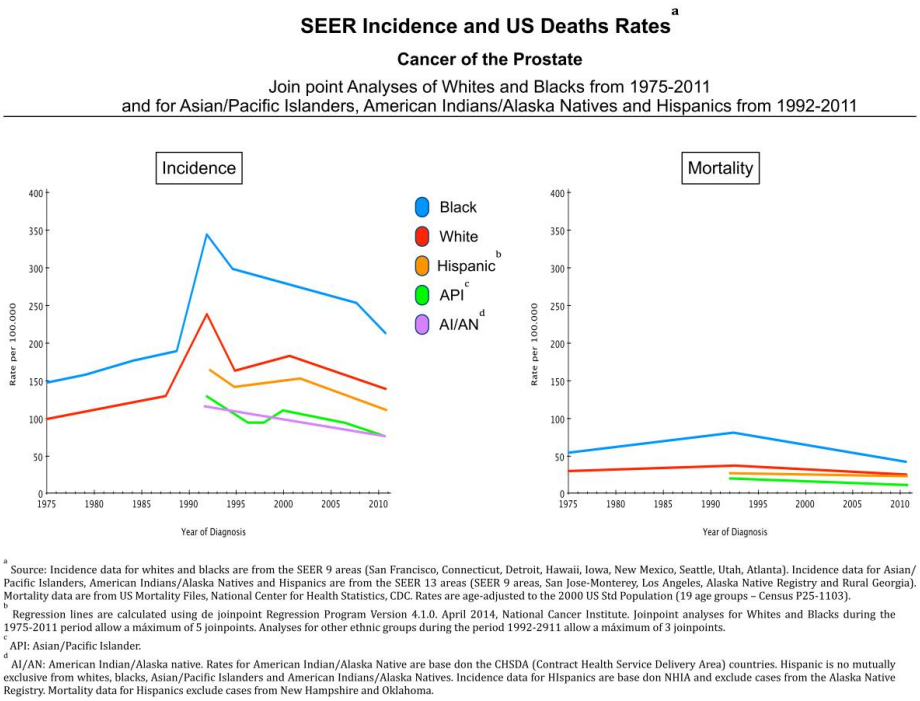

Figure 1.1 Prostate Cancer. SEER incidence and US death rates (1975-2011).

Death rates for prostate cancer have been decreasing in Northern America, Oceania, and Northern and Western Europe. This decrease has been attributed mainly to improved treatment and/or early detection, although the specific contribution of PSA testing is debated. In the other hand, in some Asian and Central and Eastern European countries mortality rates are increasing (1). Risk factors associated with economic development as increased consumption of animal fat or physical inactivity may be responsible for this trend.

1.2 Risk factors.

The only well-established risk factors for prostate cancer are increasing age, race, a family history of the disease and certain inherited genetic conditions. In fact more than 65% of all prostate cancer cases in the United States are diagnosed in men over the age of 65 years, and 97% occur in men 50 and older (2). African American men are more likely to develop prostate cancer compared with Caucasian men and are nearly 2.5 times as likely to die from the disease. Conversely, Asian men who live in Asia have the lowest risk. The reason for the high prostate cancer risk among some populations of African descent is still poorly understood, though it may in part reflect differences in genetic susceptibility (3).

Genetic studies suggest that strong familial predisposition may be responsible for 5%-10% of all prostate cancers. Compared with men that have no familiy history, those with one first-degree relative with prostate cancer have twice the risk; this risk is five times greater in men with two first-degree relatives affected. Germline mutations in the BCRA gene increase not only the risk of prostate cancer, but a more agressive disease with poor clinical outcome (4). Other studies suggest that a diet high in processed meat or dairy foods may increase risk, that obesity increases the risk of aggressive prostate cancer and that smoking is associated with prostate cancer death, but not incidence.

Prevention and early detection.

Lifestyle modifications such as smoking cessation, exercise and weight control could be contribute to reduce the risk of developing prostate cancer. Early detection of prostate cancer by prostate-specific antigen screening is controversial, because it is difficult to establish if early diagnosis is related to a benefit in overall survival, and side effects associated with a possible treatment are not negligible (5). Changes in PSA velocity or PSA doubling time, frequency of screening, and the use of other biomarkers have the

potential to minimise the overdiagnosis associated with PSA screening. Several new biomarkers for individuals with raised PSA concentrations or those diagnosed with prostate cancer will try to distinguish the more aggressive subtypes of prostate cancer and can help select in which tumors treatment should start early and in which patients the onset of therapy could be delayed.

Several pharmacological agents such as aspirin could prevent development of prostate cancer. Chemoprevention studies with 5α-reductase inhibitors (finasteride and dutasteride) are based in the effect of this compunds to reduce the amount of certain male hormones in the body (6,7) . Both drugs have been found to lower the risk of prostate cancer by 25% in large clinical trials, but its use is controversial due to: 1) a possible effect on the occurrence of more aggressive prostate cancer; 2) an increase in side effects and 3) no survival impact. Neither finasteride nor dutasteride are approved for the prevention of prostate cancer at this time.

PSA screening has played a critical role in the downward migration of prostate cancer stage seen over the past decades. The risk of prostate cancer increases with increasing PSA, but there is no level of PSA below which the risk of prostate cancer can be eliminated. Besides the difficulty of finding a PSA cutoff to discriminate malignant tumors, many doctors argue that the benefits of early detection are, at best, moderate, and that early detection could results in overdetection of a disease that would not be a problem for the patient if undetected or untreated (8). Besides false-positives results could contribute to patient anxiety and the increased costs or complications associated with unnecessary biopsies.

So in the absence of benefits in survival or quality of life it is difficult to decide on whether to make early detection of prostate cancer. Men should consider their personal risk factors and discuss them with their doctors before making a decision on whether to perform screening with PSA. Men at high risk of developing prostate cancer (African Americans or men with a close relative diagnosed with prostate cancer before the age of 65) should consider screening beginning at age 45.

1.3 Diagnosis and staging.

Signs and symptoms.

Typically, prostate cancer at an early stage causes no symptoms. However, more advanced prostate cancer can often cause symptoms such as:

- Urinary discomfort, including slow or weak urinary stream.
- Dysuria, urinary frequency and nocturia.

These symptoms can also occur in cases such as benign prostatic hypertrophy. Hematuria is also characteristic and sometimes patients may experience erectile dysfunction.

In later stages, with locally advanced or metastatic disease patients may experience pain in the pelvis, hips, spine, rib or other areas due to the spread of cancer to the bones, lymph nodes or other organs. A serious complication is spinal cord compression, which usually present with pain in spine, numbness in legs or feet, or even paraplegia and urinary incontinence.

Anatomy and histology.

The prostate lies below the bladder and encompasses the prostatic urethra (Figure 1.2). It is surrounded by a capsule and is separated from the rectum by the Denonvilliers aponeurosis. The blood supply to the base of the bladder and prostate comes from the vesicoprostatic artery, wich is derived from the internal iliac. The neurovascular bundle lies on either side of the prostate on the rectum. It is derived from the pelvic plexus and is important for erectile function.

Prostatic tumor originates in the peripheral zone of the gland, and then grows to invade the capsule, seminal vesicles and periprosthetic tissues. Spread to the central area is late and can cause urinary obstruction.

Histological diagnosis is based in performing prostate biopsy. The system used for classifying the histologic characteristics of prostate cancer is the Gleason scoring system, which uses the glandular architecture within the tumor (Figure 1.3) (9). The predominant pattern and the second-most common pattern are each given a grade of between 1 and 5 (1=more differentiated; 5=undifferentiated). The Gleason score is the sum of these 2 grades. Scoring based on the 2 most common patterns is an attempt to factor in the considerable heterogeneity within cases of prostate cancer. In addition, this scoring method was found to be superior for predicting disease outcomes compared with using the individual grades alone.

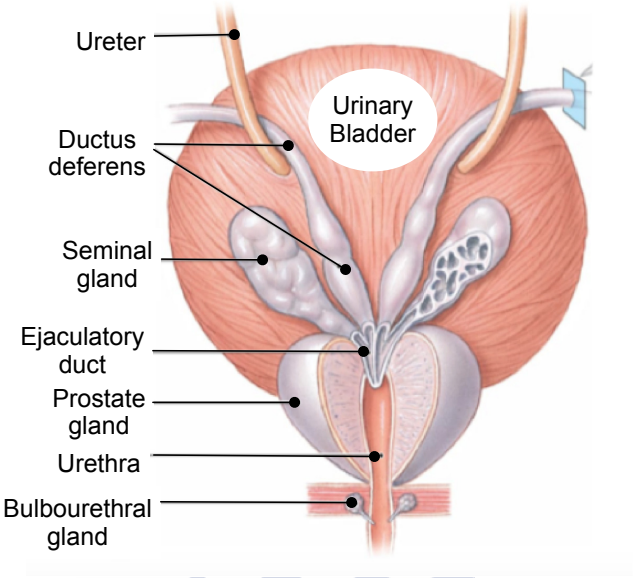

Figure 1.2. Anatomy of the prostate.

Higher Gleason grade is directly related to a number of histopathological end points, including lymphovascular space invasion by carcinoma, tumor size, positive surgical margins and pathological stage, including risk of extraprostatic extension and metastasis.

Despite revisions in 2005 and 2014, the Gleason prostate cancer grading system still has major deficiencies. Epstein et al have proposed a new prostate cancer (PCa) grading system with these possible benefits: more accurate grade stratification than current systems, simplified grading system of five grades (lowest grade is 1, as opposed to 6) with the potential to reduce overtreatment of PCa (10).

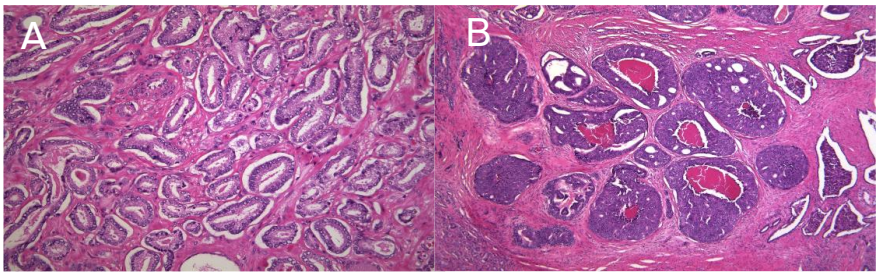

Figure 1.3. Gleason score. A. Gleason score 3, hematoxylin-eosin (HE), x10. B. Gleason score 5, hematoxylin-eosin, x10.

Histological types of prostate carcinoma are:

- Epithelial tumors: Adenocarcinoma, small cell, transitional cell, clear cell, carcinosarcoma.
- Mesenchymal malignant tumor <0.3%: Rhabdomyosarcoma, Leioimiosarcoma.
- Lymphomas.
- Prostate metastases: lymphoma, leukemia, lung adenocarcinoma, melanoma, seminoma, malignant rhabdoid tumor.

Adenocarcinoma is the most common histologic type and comprises 95% of cases of prostate cancer (Figure 1.4). It originates in the secretory cells of the acini and the ductus and it is usually multifocal and heterogeneous. More than 75% of the tumors appear in the peripheral zone. This produce a change in consistency, wich can be felt during rectal exploration/palpation. Adenocarcinoma has an infiltrative growth pattern (papillary, cribriform, comedo or acinar).

Most adenocarcinomas are of the acinar type, which are usually referred to as prostate carcinomas. More than 1% of prostate carcinoma are made up of other variants that often have a poor prognosis such as ductal carcinoma, mucinous carcinoma, signet ring cell carcinoma and small cell carcinoma. Neuroendocrine cells are one of the epithelial populations in the prostate. Neuroendocrine differentiation (NED) has been observed in prostate cancer. In addition to small cell neuroendocrine carcinomas and carcinoid tumors of the prostate, prostatic adenocarcinomas may have NED.

Diagnostic test.

Prostate tumor diagnosis is based on three elements: elevated PSA, suspicious digital rectal examination (DRE) and prostate biopsy.

PSA. Serum PSA is a protein body-specific but not cancer-specific, which explains the limitations of their use in the diagnosis of prostate cancer. However, it is the most useful tumor marker for diagnosis and monitoring of prostate cancer. Higher PSA levels correlate with higher probability of detecting prostate cancer, but there is not a lower threshold that allows rule out malignancy (7). The "normal" PSA values are between 0 and 4.0 ng / ml (low probability of cancer, especially if regular DRE); PSA> 10.0 ng / ml is highly suggestive of cancer. In patientes with levels between 4.0-10.0 ng / ml diagnoses of benign and malignant conditions overlap.

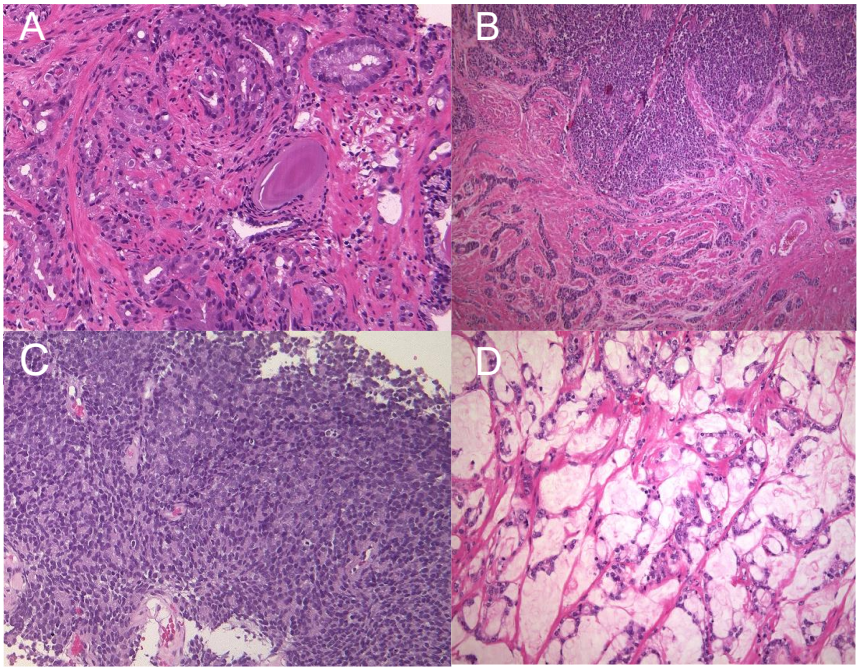

Figure 1.4. Prostate cancer histology. A. Prostate adenocarcinoma (acinar type), Gleason score 4+4, HE, x10. B. Basaloid pattern prostate adenocarcinoma, HE, x10. C. Neuroendocrine prostate carcinoma HE, x10. D. Prostate adenocarcinoma (mucinous pattern), HE, x20.

The percentage of measured PSA existing in the free form (free:total PSA ratio) is useful in assessing the risk of prostate cancer in patients with borderline or moderately increased total PSA (4.0-10.0 ng/mL) and has been used to help select men who should have follow-up prostate biopsy. Usually 10-40% of the total PSA circulates as free: PSA free/total <0.10 is associated with cancer but also should be used with caution because it

is affected by other factors.

Cancer is a growth process and it is natural that we should be concerned with how PSA changes over time. Such change is measured by PSA velocity or PSA doubling time, described in general as “PSA kinetics”. PSA velocity is given in ng/ml/year and can be thought of as a prediction: for example, a patient with a current PSA of 4 ng/ml and a PSA velocity of 0.5 ng/ml/year would be expected to have a PSA of 4.5 ng/ml in 12 months time. PSA doubling time is the number of months it would take for PSA to increase two-fold. Other methods have been proposed to increase the specificity of PSA, including age-specific PSA reference ranges and PSA density.

Digital rectal examination.

Digital rectal examination is physician dependent, and serial examinations are best. Various factors are considered when a DRE is performed. A nodule is important, but findings such as asymmetry, difference in texture and bogginess are important clues to the patient's condition and should be considered in conjunction with the PSA level. Change in texture over time can offer important clues about the need for intervention.

This maneuver has low sensitivity and specificity but improves when combined with an elevated PSA>2 ng/mL (positive predictive value (VPP): 5-30%) (11). The finding of asymmetric areas of induration or palpable nodules do suspect a malignancy and requires performing a prostate biopsy regardless of the value of PSA and especially in patients over 45 years of age with other risk factors for the disease (11). DRE can detect tumors in the lateral and posterior surface of the prostate gland but it´s not useful for 25-35% of tumors located in other locations, small tumors, T1 or non-palpable tumors. A pathological DRE is usually predictive of more aggressive prostate neoplasia (Gleason ≥ 7).

In most patients who are diagnosed with prostate cancer, however, DRE results are normal and the PSA readings are abnormal.

Prostate Biopsy.

The number of biopsies that should be performed is debated. Sextant- versus 12 or 18-core biopsy protocols are published in the literature. The 12- or 18-core protocols yield more specimens from the lateral regions and usually sample the transition zone (11). Several studies have demonstrated an increase in the cancer detection rate, but others have not.

The pathology report should include the maximum extent of the cancer, Gleason dominant pattern and higher grade (modified Gleason) to predict the pathological stage and the progression free survival (PFS).

The pathology report of a prostate biopsy should contain (11):

- Gleason score.
- Number of positive samples.
- Percentage of cancer in positive samples.
- Perineural invasion and extraprostatic extension.
- Accurate histologic subtype.
- Presence or absence of intraductal carcinoma.

In patients with a persistently elevated PSA level in the face of negative biopsy results, the literature supports repeating the biopsy once or twice. Among cancer cases, 31% were detected on repeated biopsy and 39% were detected if the PSA value was greater than 20 ng/mL.

Staging.

The staging system for prostate cancer is based on TNM AJCC but also takes into account the serum PSA and Gleason score (Table 1.1). Localized disease should be classified into prognostic groups for decision-making (12). A predictive model determines the probability of 5-year biochemical control after local treatment (90%, 60% and 30% respectively):

- Low risk: PSA <10 ng /ml, Gleason score ≤ 6 and T1c or T2a.
- Intermediate risk: PSA 10-20 ng /ml, Gleason 7 and T2b.
- High risk: PSA> 20 ng / ml, Gleason score ≥ 8 and T2c.

Staging of the disease is determined by various factors such as life expectancy, comorbidities, symptoms, extent of tumor according to the DRE, PSA level and Gleason score. Asymptomatic patients with favorable risk and life expectancy less than 10 years will not receive treatment and they dont need further testing.

Men with PSA levels above 10 ng/mL, Gleason score 7 or higher, or physical findings that suggest stage T3 disease should undergo a staging computed tomography (CT) scan and a bone scan. CT scan can be used to evaluate extension into the bladder and lymph nodes.

TNM staging System for prostate cancer

Primary Tumor (T)
Clinical

TX		**Primary tumor cannot be assessed**
T0		**No evidence of primary tumor**
T1		**Clinically inapparent tumor neither palpable nor visible for imaging**
	T1a	**Tumor incidental histologic finding in 5% or less of tissue resected**
	T1b	**Tumor incidental histologic finding in more than 5% of tissue resected**
	T1c	**Tumor identified by needle biopsy (e.g.,becasuse of elevated PSA)**
T2		**Tumor confined within prostate***
	T2a	**Tumor involves one-half of one lobe or less**
	T2b	**Tumor involves more than one-half of one lobe but not both lobes**
	T2c	**Tumor involves both lobes**
T3		**Tumor extends through the prostatic capsule****
	T3a	**Extracapsular extension (unilatera or bilaterla)**
	T3b	**Tumor invades the seminal vesicle(s)**
T4		**Tumor is fixed or invades adjacent structures other than seminal vesicles: bladder, levator muscles, and/or pelvic wall**

Patological (pT)

pT2		**Organ confined**
	pT2a	**Unilateral, involving one-half one side or less**
	pT2b	**Unilateral, involving more than one-half of one side but not both sides**
pT3		**Extraprostatic extension**
	pT3a	**Extraprostatic extension or microscopic invasion of the bladder neck**
	pT3b	**Seminal vesicle invasion**
pT4		**Invasion of bladder, rectum**

***Note: There is no pathologic T1 classification**
****Note: Positive surgical margin should be indicated by an R1 descriptor (residual microscopic disease)**

Regional Lymph Nodes (N)
Clinical

Nx	**Regional lymph nodes were not assessed**
N0	**No regional lymph node metastases**
N1	**Metastasis in regional lmph node(s)**
Pathologic	
PNX	**Regional nodes not sampled**
PN0	**No positive regional nodes**
pN1	**Metastases in regional node(s)**

Distant metastasis (M)*

M0		**No distant metastasis**
M1		**Distant metastasis**
	M1a	**Non-regional lymph node(s)**
	M1b	**Bone(s)**
	M1c	**Other site(s) with or with or without bone disease**

ANATOMIC STAGE/PROGNOSTIC GROUPS*

Group	T	N	M	PSA	Gleason
I	T1a-c	N0	M0	PSA<10	Gleason ≤6
	T2a	N0	M0	PSA<10	Gleason ≤6
	T1-2a	NO	M0	PSA X	Gleason X
IIA	T1a-c	N0	M0	PSA<20	Gleason 7
	T1a-c	N0	M0	PSA<20	Gleason ≤6
	T2a	N0	M0	PSA<20	Gleason ≤7
	T2b	N0	M0	PSA<20	Gleason ≤7
	T2b	N0	M0	PSA X	Gleason X
IIB	T2c	N0	M0	ANY PSA	Any Gleason
	T1-2	N0	M0	PSA ≥20	Any Gleason
	T1-2	N0	M0	Any PSA	Gleason ≥8
III	T3a-b	N0	M0	Any PSA	Any Gleason
IV	T4	N0	M0	Any PSA	Any Gleason
	Any T	N1	M0	Any PSA	Any Gleason
	Any T	Any N	M1	Any PSA	Any Gleason

Table 1.1. TNM staging system for prostate cancer.** *Note: When either PSA or Gleason is not available, group should be determined by T stage and/or either PSA or Gleason as available.
** Adapted from NCCN guideliness.

Magnetic resonance imaging (MRI) increases the accuracy in detecting extracapsular extension and seminal vesicles infiltration and can be useful in the selection of candidates for local treatment (PPV 70%) , and for the preservation of the nerve bundle in the radical surgery patients (Figure 1.5 and 1.6) (13). Dynamic, contrast-enhanced MRI and MR spectroscopic imaging are complementary in local staging, but their use is currently limited to selected centers. Diffusion-weighted MRI appears to improve detection of transition-zone prostate cancer.

Nodal staging should only be done if it will influence the therapeutic decision (not necessary in PSA <20 ng/mL, ≤T2a, Gleason ≤6). However patients with elevated PSA, T2b-T3 tumors, poorly differentiated or with perineural invasion are at high risk of lymph node invasion. CT scan shows superiority to MRI in the detection of nodal invasion, and is quite specific (90-95%) to high-risk patients. CT-guided FNA can be technically difficult and has many false negatives.

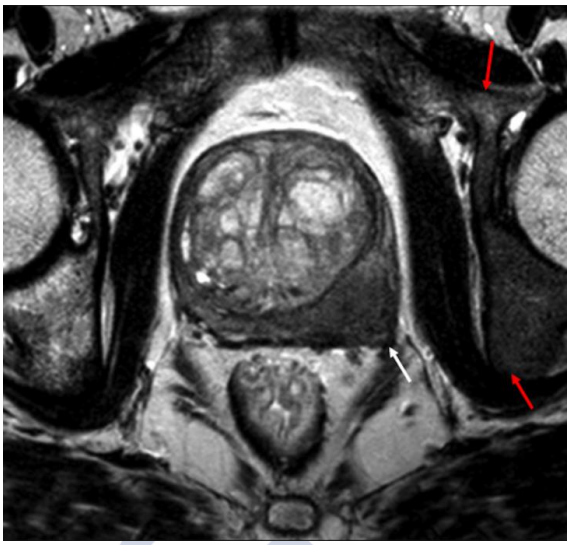

Figure 1.5. Pelvic MRI on T2 sequence. Patient with suspected prostate carcinoma (PSA 18). Pelvic MRI on T2 sequence shows a neoplasm invading the neurovascular left band (white arrow) and alteration of the bone signal (red arrows).

The gold-standard for nodal staging is open or laparoscopic lymphadenectomy and is usually reserved for patients at high risk of nodal spread. It is not recommended for those who are going to be treated with radiotherapy. C-choline PET has low sensitivity for detecting lymph node involvement.

Prostate cancer has a predilection for bone, affecting 85% of patients who die from the disease. Other common metastatic sites are: lymph nodes, lung, liver, brain and skin. PSA can be useful to determine which tests should be performed in the staging. PSA values > 100 ng / mL predict distant disease with a PPV of 100%

In case of suspected metastasis or when it´s necessary to complete staging in high risk patients abdominopelvic CT it is recommended to rule out locoregional involvement and Rx chest to rule out pulmonary metastases. Spine MRI is used to study malignant vertebral compression in patients with vertebral pain or neurological symptoms.

MRI is superior to bone scanning in evaluating bone metastasis but their use as routine total-body surveys is not yet widespread (Figure 1.7) (14). Instead, it is used to determine the etiology of questionable lesions found on bone scans.

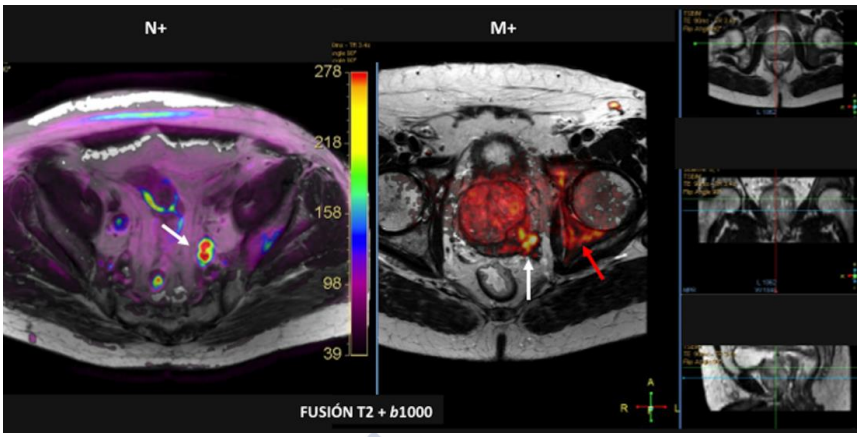

Figure 1.6. Pelvic MRI (T2 and diffusion). Fusion of sequences T2 and diffusion imaging showing high b value. Color map shows malignant presacral lymph nodes and a left iliac lymph node positive (white arrow, right image). Diffusion is restricted in prostate tumor (white arrow, center) and bone involvement is confirmed (red arrow). Stage cT3cN + CM1 (bone).

ProstaScint scanning involves the use of a murine monoclonal antibody that reacts with prostate-specific membrane antigen to identify cancer in the prostate and in metastatic deposits. F-fluorodeoxyglucose (18F-FDG) currently has no indication in the staging of prostate cancer. However the C-choline PET is useful in assessing local relapse of prostate cancer when a local salvage therapy is assessed.

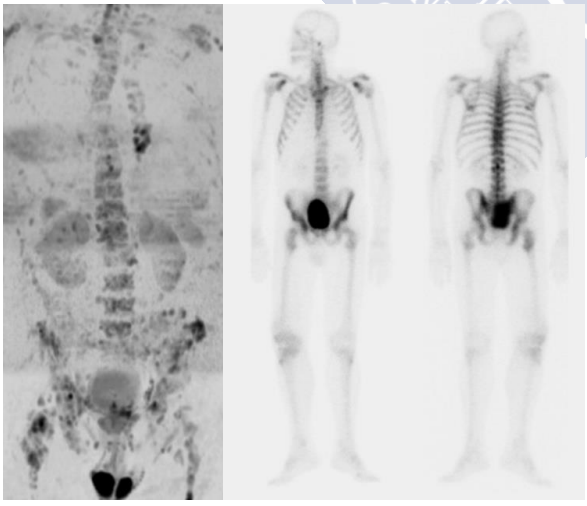

Figure 1.7. Assessment of bone disease in patients with prostate cancer. 61 years old man. PSA 73 ng/mL, alkaline phosphatase 1125 UI/L. A bone scan was negative. Whole-body MRI showed multiple bone metastases.

Prognostic factors and biomarkers. Molecular testing.

In the therapeutic management of patients with prostate cancer factors such as life expectancy and biological characteristics of the tumor should be assessed. Prostate cancers are best characterized by clinical stage determined by DRE, Gleason score in the biopsy specimen and serum PSA level. The more clinically relevant information that is used in the calculation of time to PSA failure, the more accurate the result.

Several approaches based on clinical factors are used to assess the prognosis of prostate cancer patients. The risk stratification schemes most commonly used are those of D'Amico and NCCN (15-18). Stratification helps clinicians to distinguish between tumors with better and worse prognosis and so take treatment decisions based on this risk (Figure 1.8).

The Partin tables were the first to achieve widespread use for counseling men with clinically localized prostate cancer (19). This tables give the probability (95% confidence intervals) that a patient with a certain clinical stage, Gleason score and PSA will have a cancer of each pathologic stage. Nomograms can be used to inform treatment decision-making for men contemplating active surveillance, radical prostatectomy, neurovascular bundle preservation or omission of pelvic lymph node dissection (PLND) during radical prostatectomy, brachytherapy, or external beam radiation therapy (EBRT).

Given the non-related cancer causes of mortality, many men who sustain PSA failure will not live long enough either to develop clinical evidence of distant metastases or to die from prostate cancer. Not all PSA failures are clinically relevant, thus PSA doubling time may be a more useful measure of risk of death: men with a short PSA doubling time are at greatest risk of death.

Several genomic/proteomic tests have become commercially available in recent years. Althougth they have not been approved by the FDA and their validation is somewhat limited it could help make better clinical decisions for treating PCa patients.

The Oncotype DX was developed to test fixed paraffin-embedded tissue samples that were obtained by needle biopsy. The assay measures the expression of 12 cancer-related genes which represent four different biological pathways [cellular organization (FLNC, GSN, TPM2, and GSTM2); androgen pathway (AZGP1, KLK2, SRD5A2, and RAM13C); proliferation pathway (TPX2); and stromal response (BGN, COL1A1 and

SFRP4)] and five reference genes (used to normalize and control preanalytical and analytical variability). These genes are algorithmically combined to calculate the Genomic Prostate Score (20). The combination of GPS and NCCN criteria improves risk discrimination of PCa into very low, low and modified intermediate risk in order to select appropriate candidates for active surveillance.

The Prolaris assay produces a cell cycle progression (CCP) score from RNA expression levels of 31 genes involved in CPP that were selected because of correlation with proliferation of PCa. This molecular test directly measures tumor cell growth characteristics to stratify disease risk of progression. Cooperberger et al validated CPP in a prostatectomy cohort (21). They found that low expression is associated with a low risk of disease progression, whereas high expression is associated with increased risk of disease progression. This test may be useful to determine which patients need either close monitoring or additional therapy.

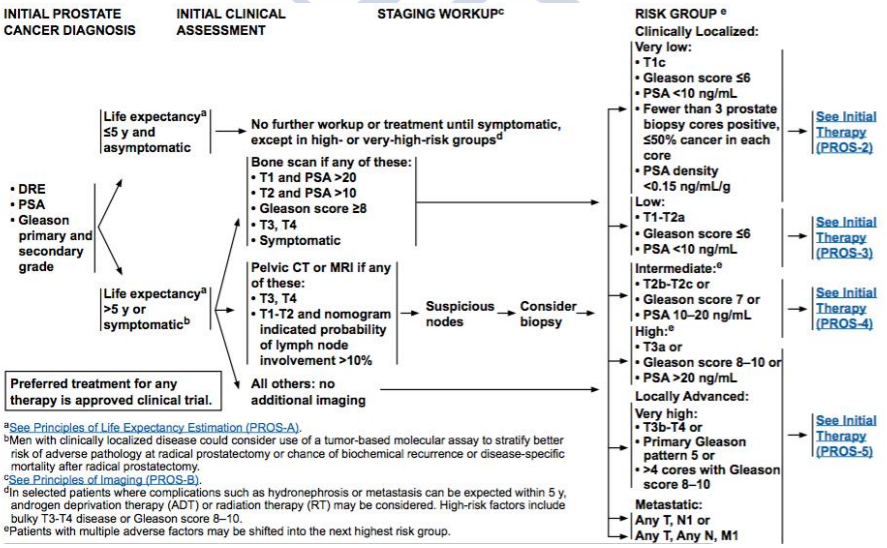

Figure 1.8. Risk stratificacion based treatment. From NCCN guideliness version 1.2015.

2. Treatment.

2.1 Non metastatic disease.

Most patients present with tumors confined to the prostate (Figure 1.9). Treatment options vary depending on age, stage, and Gleason score, as well as other medical conditions.

In *low risk prostate cancer*, the patient decide whether he prefers an active treatment (surgery, brachytherapy or external beam radiotherapy(EBRT)) versus active surveillance. Active surveillance instead of immediate treatment is appropriate for many patients, particularly men with less aggressive tumors and for older men.

Intermediate risk prostate cancer. The patient can choose from a radical prostatectomy with extended lymphadenectomy versus external beam therapy (3D CRT/intensity modulated radiotherapy (IMRT)) associated with 6 months of androgen deprivation. Brachytherapy is also an alternative in selected patients. The patient has moderate risk of erectile dysfunction regardless of the chosen method of treatment.

High risk disease. The patient should know that it is feasible that he requires a multimodal treatment. The best therapy choice should be identify in a multidisciplinary committee: clinical characteristics of the tumor, performance status and preferences of the patient shoud be considered. Options are IMRT + 2-3 years of androgen deprivation vs radical prostatectomy with extended lymphadenectomy and potential further treatments depending on the surgical margins, nodal status and biochemical evolution. Brachyterapy has a role as dose intensification in intermediate and high risk prostate cancer.

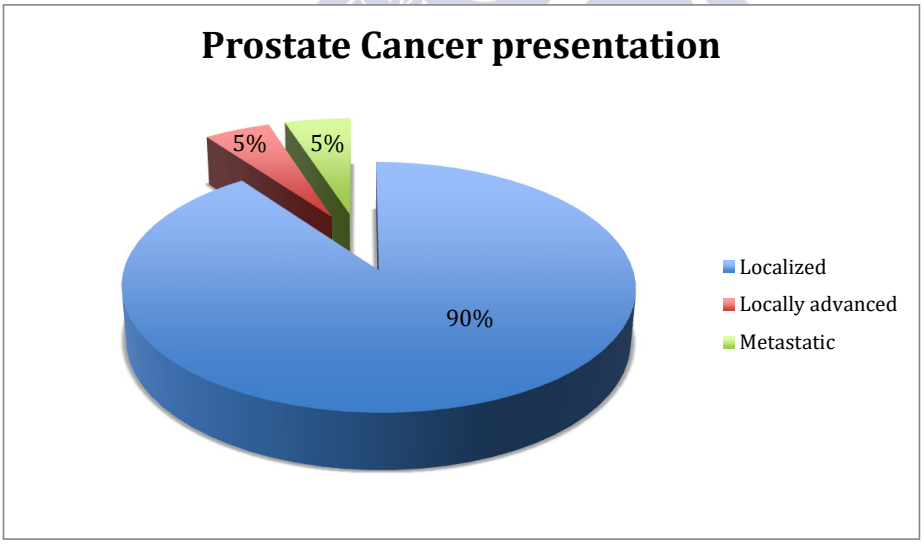

Figure 1.9. Prostate cancer presentation.

Active surveillance.

Active surveillance began as a necessity due to the overtreatment of many patients with indolent prostate tumors, recognizing them as tumors that had not conditioned the life expectancy of the patients. Active surveillance differs from watchful waiting. With watchful waiting, patients forgo close follow-up and primary treatment. With active surveillance, the physician monitors the course of the disease over time and and decide to start treatment if the disease progresses.

Active surveillance is increasingly being recommended for men with low-risk disease, which includes T1-2a disease, a Gleason score of 2-6, and a PSA level below 10 ng/mL. Progression of local disease may be indicated either by increased tumor volume, changes in the Gleason score or even by PSA doubling times.

The optimal management of men on active surveillance is evolving, and varies according to different guidelines. Monitoring typically consists of PSA testing every 3 months and repeat biopsy at 12- to 24-month intervals. Biopsy findings are the most important factor in deciding whether to pursue treatment. A rapid PSA level rise or patient choice can also prompt the physician to proceed to treatment (22).

Surgery.

Radical prostatectomy (RP) involves complete resection of the prostate and seminal gland. The goal of radical prostatectomy is the eradication of the disease preserving the quality of life of patients, and so it is only appropriate for men whose tumor is confined to the prostate. The surgical option should not be ruled exclusively by the patient's age, but because of potential perioperative morbidity it should be reserved for patients whose life expectancy is 10 years or more.

Radical prostatectomy is popularly associated with risk of incontinence and impotence. However, such complications arising from the anatomical position of the gland and so any prostate treatment involves a certain risk of injury to these structures and the development of complications. The prostate is overlapped at its distal portion with the external sphincter muscle and is involved in the dorsolateral area by neurovascular tissue responsible for erection. Prostatectomy includes the dissection of the gland regarding its neighboring structures (bladder, rectum, sphincter and neurovascular bundles) and then anastomizing the bladder to the urethra through a suture.

Minimally invasive techniques have shown only better intreoperative bleeding. The only variable that is associated with better oncological and functional results is the experience of the surgeon. Lymphadenectomy is associated with prostatectomy based on the clinical risk group of metastases: it is generally recommended when the risk of metastasis is more than 5%. For the calculation of risk is often used multivariate prediction models (23).

Stephenson and colleagues reported a low 15-year prostate cancer-specific mortality of 12% in patients who underwent radical prostatectomy (5% for low-risk patients), despite the presence of adverse clinical features. It is unclear whether the favorable prognosis is due to the effectiveness of the procedure or the low lethality of cancers detected in the PSA era (24).

The SPCG-4 trial, with a median follow-up of 15 years, showed that radical prostatectomy was associated with a reduced risk of death (HR= 0.75; 95%CI: 0.61 to 0.92) and the risk of death from prostate cancer (HR= 0.62; 95%CI: 0.44 to 0.87) (25). However the PIVOT study, published a year later, failed to reproduce these results, reducing the benefit of the RP for patients with PSA> 10 ng/ml and possibly among those with for intermediate or high risk tumors (26).

Some patients at high or very high risk may still benefit from radical prostatectomy. In an analysis of 842 men with high-Gleason sum at biopsy who underwent to radical prostatectomy a PSA concentration of >10 ng/mL, clinical stage ≥ T2b, Gleason pattern 9 or 10, increasing number of cores with high-grade cancer and >50% core involvement were predictive of unfavourable pathology (27). In men with favourable pathological findings the 10-year biochemical-free (BFS), metastasis-free (MFS) and prostate cancer-specific survival (CSS) were 31.0%, 60.9% and 74.8%, respectively. In contrast, men with unfavourable pathological findings had significantly worse 10-year BFS, MFS and CSS: 4.3%, 29.1% and 52.3%, respectively (all $p < 0.001$).

Laparoscopic and robot-assisted radical prostatectomy are used commonly and are considered comparable to conventional approaches in experienced hands. Both techniques have comparable rates of complications and the need for additional cancer therapies is similar to the classic techniques. In a study conducted by Gandaglia et al robot-assisted radical prostatectomy was associated with lower risk of blood transfusions and a slightly shorter length of stay (28).

Robot-assisted surgery is being implemented today and the question for patients considering surgical treatment of their prostate cancer is not to choose a technique, but to choose a surgeon who is an expert at a given technique, to minimize surgical complication risk (29).

Radical prostatectomy is a salvage option for patients experiencing biochemical recurrence after primary RT, but morbidity (incontinence, erectile dysfunction, and bladder neck contracture) remains significantly higher than when radical prostatectomy is used as initial therapy. Biochemical recurrence-free probability after salvage radical prostatectomy ranged from 47% to 82% at 5 years and from 28% to 53% at 10 years (30). Patient selection is important and prostatectomy should only be performed by highly experienced surgeons.

Regarding pelvic lymph node dissection (PLND), while extended lymphadenectomy is popular in Europe, standard PLND has always been preferred in the United States. An extended PLND reaches from the bifurcation of the common iliac artery superiorly to the femoral canal inferiorly; posteriorly, the obturator nerve, obturator vessels, and internal iliac artery are skeletonized. In standard PLND the internal iliac artery nodes are not removed. These approaches carry with them differing morbidities as well as the possibility of varied staging and curative advantages. A survival advantage with more extensive lymphadenectomy has been suggested by several studies, possibly due to elimination of microscopic metastases.

Radiotherapy.

Radiation therapy offers the potential for curative treatment of localized prostate cancer. It may be delivered in the form of external-beam radiation therapy or brachytherapy (insertion of radioactive seeds into the prostate gland). EBRT techniques include 3-dimensional conformal radiation therapy (3D-CRT) and intensity-modulated radiation therapy. Stereotactic body radiotherapy (SBRT) is an emerging treatment technique that delivers highly conformal, high-dose radiation in 5 or fewer treatment fractions, which are safe to administer only with precise, image-guided delivery (Figure 1.10).

Figure 1.10. External beam radiotherapy unit.

Randomized trials comparing surgery with radiation either have not yet been reported or have been abandoned because of poor accrual. In this context, most men with localized prostate cancer are encouraged to make treatment decisions based on the toxicity profiles of the treatments and personal preferences. The acute effects of radiation, defined as occurring up to 6 months after treatment, include urinary obstructive symptoms, bowel symptoms and fatigue (31,32) . Late toxicities may include sexual dysfunction, persistent bowel problems (intermittent rectal bleeding, tenesmus and urgency) and urinary obstructive symptoms. An increased risk of bladder, colon or rectum tumors can occur after prostate radiation therapy.

Doctors commonly use risk groups based on clinical and pathologic information known at the time of diagnosis to guide treatment decisions. The risk groups are commonly defined by the tumor (T)-classification, the Gleason score, and the PSA level at diagnosis (Table 1.2).

Significant progress has also been made in the use of genetic signatures from tumor tissue in providing additional prognostic information, but we need large prospective studies to know wheter these emerging tests can be used to select patients who need radical therapy, e.g radiotherapy.

The use of EBRT to the prostate for men with prostate cancer has been established by two randomized controlled trials. Both studies were done mainly in men with high-risk or locally advanced disease. The National Cancer Institute of Canada-PR3 trial assigned 1205 prostate cancer patients between 1995 and 2005 to lifelong androgen deprivation

Table 1.2. Group risk classifcation.

D´AMICO	CURRENT
Low risk	Very low risk
▪ Gleason ≤6, and	▪ T1c, and
▪ PSA <10 ng/mL, and	▪ Gleason ≤6, and
▪ ≤T2a	▪ PSA<10 ng/mL, and
	▪ PSA density <0.15 ng/mL, and
	▪ ≤2 cores positive, and
	▪ ≤50% cancer in each core
	Low risk
	≤T2a, and
	Gleason ≤6, and
	PSA< 10 ng/mL
Intermediate risk	Favorable intermediate risk
▪ Gleason 7, or	▪ T2b-T2c, or
▪ PSA 10-20 ng/mL	▪ Gleason 7, or
▪ T2b	▪ PSA 10-20 ng/mL
	Unfavorable intermediate risk
	Primary Gleason grade 4, or
	≥50% cores positive, or
	Multiple intermediate risk factors
High risk	High risk
▪ Gleason 8-10, or ≥T2c, or	▪ Gleason 8-10, or T3a, or
▪ PSA> 20 ng/mL, or	▪ PSA > 20 ng/mL
	Very high risk
	▪ ≥T3b

PSA: prostate specific antigen

therapy (luteinizing hormone-releasing hormone agonist or antagonist or bilateral orchiectomy), with or without radiotherapy (33). The addition of radiotherapy significantly reduced 7-year overall mortality from 34% to 26% (HR 0.77 [95% CI 0.61–0.98], p=0.033). The SPCG-7 trial randomly assigned 875 patients between 1996 and 2002 to hormonal treatment alone (3 months of total androgen blockade followed by flutamide alone) or to the same hormonal treatment combined with radiotherapy (34). Radiotherapy significantly improved 10 year overall mortality (30% *vs* 39%, p=0.004). Side effects of radiation therapy were mild in both studies, rectal bleeding as the most significant complication. Long-term androgen deprivation plus radical radiotherapy are now a standard of care for high-risk and locally advanced prostate cancer. As a consequence of these trials, hormone therapy as exclusive treatment is not recommended for men with localised or locally advanced disease.

Level I evidence supports the use of higher doses in the 75.6-Gy to 79.2-Gy range as a standard treatment of localized prostate cancer (35-38). Observational data suggest that IMRT reduces the risk of important late toxicities over 3DCRT, although treatment cost is increased (Figure 1.11) (39). This increase in cost has led to a call for greater comparative effectiveness research in prostate cancer in the setting of less costly options.

Moderately hypofractionated image-guided IMRT regimens (2.4–4 Gy per fraction over 4-6 weeks) have been tested in randomized trials and efficacy and toxicity have been similar to conventionally fractionated IMRT (40,41). Given the atractiviness of this approach to patient convenience and reduced cost and the successes of hypofractionated treatment in breast cancer, it is considered that this treatment modality may actually be useful (42). However, long-term efficacy results from non-inferiority trials are needed before moderate hypofractionation can confidently be widely adopted.

The same consideration applies to the case of extreme hypofractionation. A randomized phase 2 study is currently underway through the RTOG (NCT01434290) comparing five 7.25-Gy fractions with twelve 4.3-Gy fractions. In the International Standard Randomized Controlled Trial Number 45905321 open in Sweden 592 men with intermediate-risk prostate cancer will be randomized to 78 Gy in 2-Gy fractions versus 7 fractions of 6.1 Gy to a total dose of 42.7 Gy.

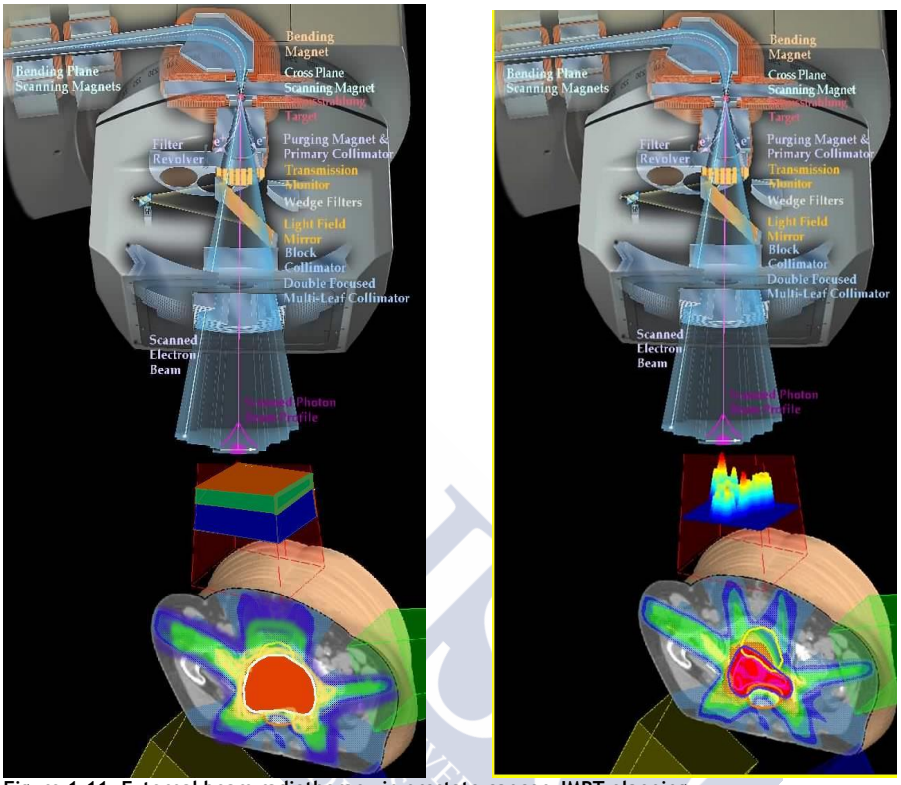

Figure 1.11. External beam radiotherapy in prostate cancer. IMRT planning.

Brachytherapy is generally used only in men with early stage PCa. Its use is also limited by other factors. For men who have had a transurethral resection of the prostate (TURP) or for those who already have urinary problems, the risk of urinary side effects may be higher. Men with large prostate glands are not good candidates for brachytherapy because it might not be possible to place the seeds into all of the correct locations.

Typically Iodline-125 or Palladium-103 are used as isotopes, and the sources are placed transperineally with ultrasound guidance (Figure 1.12). Approximately 100 of these 4-mm to 5-mm sources are used to treat the entire prostate. Low dose-rate brachytherapy has excellent results, with single-institution series reporting 10-year disease-specific survival rates greater than 95% (43).

Permanent brachytherapy as monotherapy is indicated for patients with low-risk cancers (cT1c–T2a, Gleason grade 2-6, PSA <10 ng/mL) and selected patients with low volume intermediate-risk cancers. Patients with high-risk cancers are generally considered poor

candidates for permanent brachytherapy alone. Given the good local control achieved with brachytherapy attempts have been made to combine with EBRT with or without neaodjuvant androgen deprivation therapy (ADT), but the complication rate increases (44).

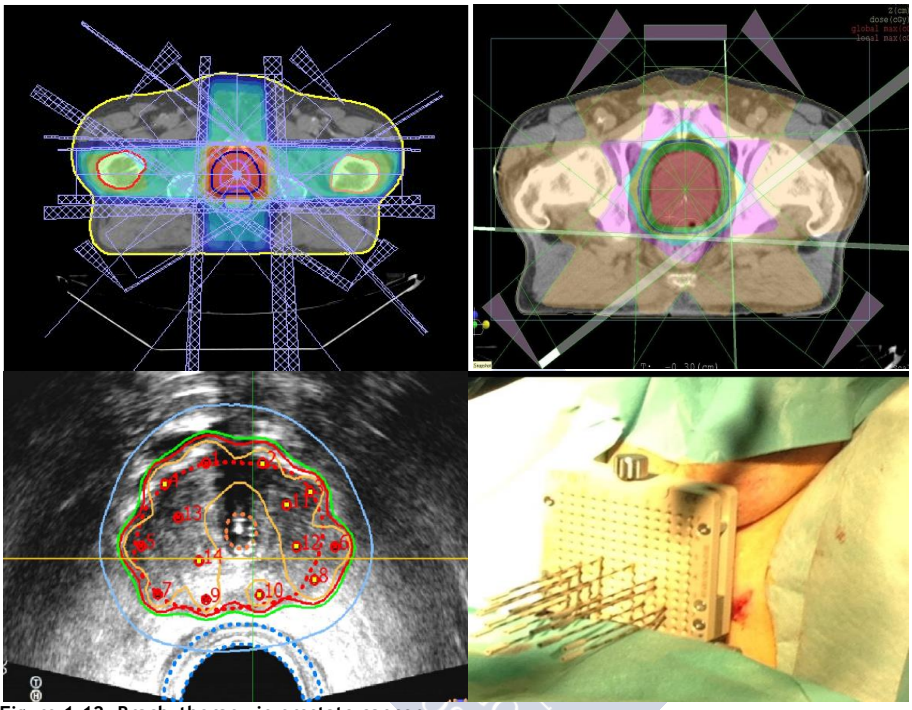

Figure 1.12. Brachytherapy in prostate cancer.

Low dose-rate brachytherapy consists of placement of permanent seed implants in the prostate. High dose-rate brachytherapy, which involves temporary insertion of a radiation source, is a newer approach that provides a "boost" dose in addition to EBRT for patients at high risk of recurrence. This approach allows for the treatment of higher risk features, such as extracapsular extension. Typically delivered over fewer than 10 fractions, several single-institutional series have demonstrated both excellent disease control and modest acute urinary and gastrointestinal side effects (45). In the RTOG 00-19 trial 45 Gy were delivered with external beam radiation followed by brachytherapy to an additional 108 Gy (46). With 4 years of follow-up, 15% of patients had a grade 3 or higher GU or GI toxicity, and biochemical control was similar to that reported using external beam radiation alone.

Androgen deprivation is used as an adjuvant to external-beam radiotherapy for higher-risk disease (Table 1.3). In a randomised trial of 415 patients with locally advanced disease, 3 years of androgen deprivation added to radiotherapy improved 10 year overall survival from 40% to 58% (HR 0.60, 95% CI 0.45–0.80; p=0.0004) (47). Due to the excellent prognosis of low risk, the use of ADT offers no advantages in this group of patients. The optimum duration of adjuvant androgen deprivation is uncertain.

TROG 96.01 compared none, 3 months or 6 months of neoadjuvant androgen deprivation in 818 men undergoing radiotherapy for localised and locally advanced disease (48). The use of 6 months, not 3 months, neoadjuvant androgen-deprivation significantly improved overall mortality (43% versus 29% at 10 years, HR 0.63, 95% CI 0.48–0.83; p=0.0008). So 6 months should be considered the minimum duration of adjuvant treatment. EORTC 22961 randomised 970 men between 6 months and 3 years of adjuvant treatment (49). Overall mortality at 5 years was 19% and 15% (HR 1.42, 95% CI 1.09–1.85), respectively. This survival benefit comes at the cost of a substantial prolongation of treatment-related morbidity.

Table 1.3. Influential phase 3 trials for radiotherapy in local prostate cancer.

	Patients	Comparison	Overall survival outcome	HR(95%CI)	P value
Trials of hormone therapy with or without radical radiotherapy					
SPCG-7	875	Hormone therapy plus EBRT vs hormone therapy	70% vs 61% at 10 years	--	0.004
NCIC PR3	1205	Hormone therapy plus EBRT vs hormone therapy	74% vs 66% at 7 years	0.77 (0.61-0.98)	0.033
Trials of radical raditoherapy with ot without adjuvant hormone therapy					
EORTC 22863	415	EEBRT plus 3 years of hormone therapy vs EBRT	58% vs 40% at 10 years	0.60 (0.45-0.80)	0.0004
RTOG 8531	977	EBRT plus lifelong hormone therapy vs EBRT	49% vs 39% at 10 years	--	0.002
TROG 9601	537	EBRT plus 6 months of hormone therapy vs EBRT	70.8% vs 57.5% at 10 years	0.63(0.48-0.83)	0.0008
EBRT= external-beam radiaton therapy					

2.2 Metastatic disease.

Despite the excellent results achieved with surgery and radiotherapy unfortunately, approximately 30% of with prostate cancer patients will develop advanced disease. Metastatic prostate cancer patients are treated with hormonal therapy, chemotherapy, radiation therapy and/or other treatments.

Hormone treatment may control advanced prostate cancer for long periods by shrinking the size or limiting tumor growth, thus helping to relieve pain and other symptoms. Androgen deprivation therapy achieves disease control in about 90% of these men, for a median of 18 months, but most of them eventually will develop progressive disease, a status called castration-resistant prostate cancer (CRPC).

In 2004 the combination of docetaxel and prednisone demonstrated an improvement in overall survival (OS) (50,51). Since then, several new treatments such as immunotherapy, new hormonal manipulations, modern chemotherapy agents and bone-targeted therapies have also demonstrated an improvement in OS in clinical trials (Figure 1.13).

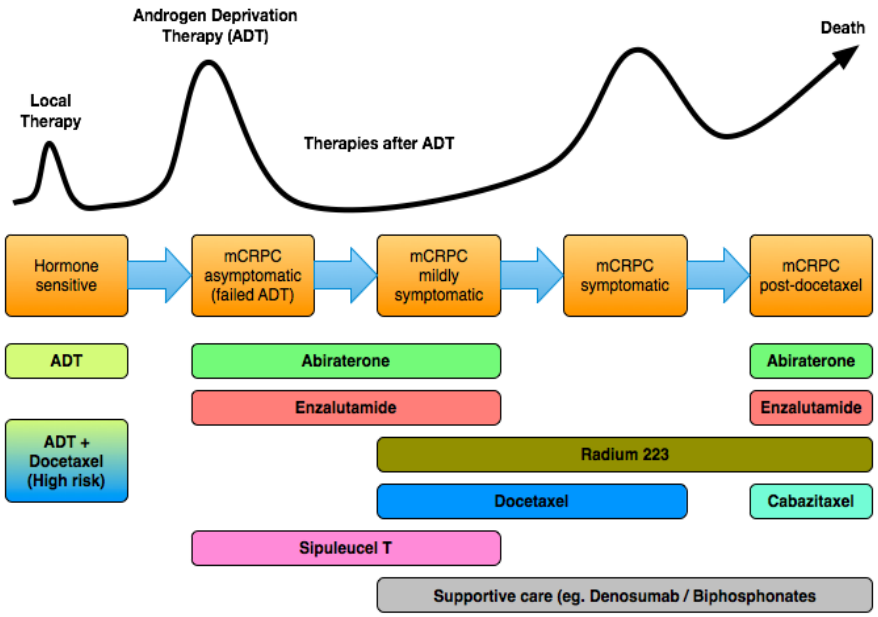

Figure 1.13. Metastatic prostate cancer treatment.

Castration sensitive metastatic prostate cancer.

Hormonal treatment.

Androgen deprivation is considered the primary approach to the treatment of metastatic prostate cancer (52). Prior to the development of newer therapies, overall survival for patients with metastatic prostate cancer ranged from 24-36 months. In recent years the introduction of new agents have allowed prolong survival after failure of hormonal treatment.

ADT can be accomplished using bilateral orchiectomy (surgical castration) or a luteinizing hormone-releasing hormone (LHRH) agonist or antagonist (medical castration), which are equally effective. The addition of an antiandrogen to LHRH agonist treatment can minimize the risk of the flare response (ie, temporary rise in testosterone levels) that can occur with LHRH treatment (53). No initial flare is associated with LHRH antagonists and no coadministration of anti-androgen is necessary.

Medical or surgical castration combined with an antiandrogen is known as **combined androgen blockade** (CAB). No prospective randomized studies have demonstrated a survival advantage with CAB over the serial use of an LHRH agonist and an anti-androgen (54). Meta-analysis data suggest that bicalutamide may provide an incremental relative improvement in overall survival by 5% to 20% over LHRH agonist monotherapy, but a clinical trial is necessary to test this hypothesis (55). One explanation for many of the negative studies is the antiandrogen withdrawal phenomenon, in which a tumor that has started to grow despite antiandrogen treatment regresses when antiandrogen treatment is stopped. If the PSA level begins to rise in a patient who is receiving CAB, the antiandrogen should be discontinued before other therapy is initiated. Generally, 1-2 months are needed following antiandrogen withdrawal to see whether the patient will improve. The optimal interval varies with different antiandrogens. Antiandrogen monotherapy appears to be less effective than medical or surgical castration and is not recommended as primary ADT.

In the years following the introduction of hormone therapy for prostate cancer it was debated whether the late onset of ADT could maintain survival benefit delaying the onset of side effects (56). Laboratory studies developed with LHRH antagonist and LHRH agonists demonstrated that early hormone therapy does not confer early

resistance. Moreover, clinical trials found that it provided significantly longer survival with fewer complications (eg, pathologic fractures, spinal cord compression, ureteral obstruction) than did deferred treatment (57,58).

Intermittent androgen suppression has been assessed in prospective, randomized studies as a possible means of minimizing the side effects of ADT. Two phase III trials found that intermittent androgen suppression resulted in better quality of life and it was noninferior to continuous therapy with respect to overall survival (59,60). However, according to a large study of 770 men treated with intermittent therapy and 765 men treated with continuous therapy survival may be shorter when androgen deprivation therapy is given intermittently rather than continuously (average survival, 5.1 vs 5.8 years, a 10% higher relative risk for death). Intermittent therapy was associated with better erectile function and mental health at month 3 but not thereafter.

Docetaxel in hormone-sensitive prostate cancer.

Two studies have tested the role of docetaxel in addition to ADT in patients with hormone-sensitive prostate cancer. The GETUG-AFU 15 phase III trial randomized 385 patients with metastatic castration sensitive prostate cancer to receive ADT or ADT plus docetaxel (75 mg/m2 iv every 3 weeks, up to 9 cycles) (61). After a median follow-up 50 months, median OS was 58.9 months for the ADT plus docetaxel arm, versus 54.2 months for patients receiving only ADT (HR 1.01, 95% CI 0.75-1.36). However, secondary objectives were met: clinical and biochemical progression free survival were statistically significant higher for the docetaxel plus ADT arm.

The E3805 (CHAARTED) study enrolled 790 mCRPC patients between July 2006 and November 2012. They were randomized to receive ADT versus ADT plus docetaxel. In this case, the primary endpoint was met: after a median follow-up of 29 months, OS was statistically significant better for the combination arm (57.6 versus 44.0 months, HR 0.61, 95% CI 0.47-0.80). Patients with high metastatic disease burden (visceral metastases and/or four or more bone metastases with at least one beyond the axial skeleton) increased their survival by 17 months (49.2 versus 32.2 months, HR 0.60, 95% CI 0.45-0.81). In patients with a low metastatic disease burden, median OS has not been reached yet (HR 0.63, 95% CI 0.34-1.17). Secondary objectives (PSA <0.2 ng/mL at 6 and 12 months, median time to become castration resistant and median

clinical and biological PFS were also statistically significant better for the ADT plus docetaxel arm (62).

The differences observed between the two studies may be related to the different populations included (more high-risk patients in the study CHAARTED), as well as variations in the use of treatments in subsequent lines. Results from the Stampede study have been shared at ASCO 2015, showing a clinical and statistically significant benefit in survival of 10 months from adding docetaxel to ADT (63). In this study, four arms were tested: hormone therapy for > =3 years, hormone therapy plus docetaxel, hormone therapy plus docetaxel and zoledronic acid, and hormone therapy plus zoledronic acid alone (no benefit from adding zoledronic acid was observed).

Castration resistant metastatic prostate cancer.

Castration resistant prostate cancer is defined as disease progression (increase in PSA in serum, new clinical metastases, or progression of existing metastases) despite the administration of ADT. The Prostate Cancer Clinical Trials Working Group 2 (PCWG2) defines CRPC as patients with serum castration levels of testosterone (testosterone <50 ng/dL or <1.7 nmol/L), PSA progression and/or clinical progression to castration, or progression despite anti-androgen withdrawal for at least 4–6 weeks (64).

Since the introduction of docetaxel in the treatment of mCRPC, designed randomized trials with new drugs have established two large categories: chemonaive and chemotherapy pretreated patients. The following sections briefly discusses the main therapeutic options currently available (Table 1.4).

Hormonal treatment. New agents.

When disease progresses, discontinuation of LHRH analogs therapy can result in an increase in serum testosterone and, thus, promoting tumor growth. There are currently no prospective trials demonstrating the impact of discontinuing ADT, and retrospective analysis provide conflicting results (65,66) . Despite the lack of strong clinical data, the current recommendation is to continue androgen suppression in all patients as an eligibility criterion for phase III trials in the androgen-resistant setting.

Antiandrogen withdrawal should be considered in most patients with CRPC, except in symptomatic patients or in those who are showing rapid and aggressive progression (67). After the failure of the classical hormonal treatment we can consider for using

different therapies introduced in recent years (Figure 1.14). Here we focus on the two drugs with more data of phase III trials: abiraterone and enzalutamide.

Table 1.4. Phase III trials evaluating agents which have been demonstrated activity in patients with mCRPC.

Study	N	Status	Regimen	rPFS, months	p	OS, months	p
TAX 327	1006	CT-naïve	Docetaxel+prednisone vs. mitoxantrone+prednisone	NR	NR	18.9 vs. 16.5; HR: 0.76	0.009
COU-AA-302	1088	Asymptomatic CT-naïve	Abiraterone+prednisone vs. placebo+prednisone	NR vs. 8.3; HR: 0.43	<0.0001	NR vs. 27.2; HR: 0.75	0.0097
PREVAIL	1717	Asymptomatic CT-naïve	Enzalutamide vs. placebo	13.8 vs. 3.9; HR: 0.18	<0.0001	32.4 vs. 30.2; HR: 0.70	<0.0001
ALSYMPCA	922	Pre and post-docetaxel	Radium 223 vs. placebo	15.6 vs. 9.8; HR: 0.66	<0.001	14.9 vs. 11.3; HR: 0.695	0.00007
IMPACT	512	Pre and post-docetaxel	Sipuleucel T vs. placebo	3.7 vs 3.6; HR: 0.95	NS	25.8 vs. 21.7; HR: 0.78	0.03
TROPIC	755	Post-docetaxel	Cabazitaxel+prednisone vs. mitoxantrone+prednisone	2.8 vs. 1.4; HR: 0.74	<0.0001	15.1 vs. 12.7; HR: 0.70	<0.0001
COU-AA-301	1195	Post-docetaxel	Abiraterone+prednisone vs. placebo+prednisone	5.6 vs. 3.6; HR: 0.67	<0.001	15.8 vs. 11.2; HR: 0.74	<0.0001
AFFIRM	1199	Post-docetaxel	Enzalutamide vs. placebo	8.3 vs. 2.9; HR: 0.4	<0.001	18.4 vs. 13.6; HR: 0.63	<0.0001
Fizazi *et al.*	1904	Pre and post-docetaxel	Denosumab vs. zoledronic acid	20.7 vs. 17.1; HR: 0.82	0.008	19.4 vs. 19.8; HR: 1.03	NS

CT: chemotherapy; HR: hazard ratio; NR: not reported; NS: not statistically significant; OS: overall survival; PFS:progression free survival; rPFS: radiological progression free survival; tSRE: time to first skeletal-related event.

Abiraterone acetate (AA) is a selective, irreversible, and potent inhibitor of 17-[alpha]-hydroxylase/17,20-lyase (CYP17), a critical enzyme in testosterone synthesis. This agent can block androgen synthesis developed in the adrenal glands, testicles and prostate cancer cells, to reduce blood testosterone levels below detectable limits (< 1 ng/dL). Recently, abiraterone has demonstrated activity in castration resistant prostate cancer patients prior to and after docetaxel administration.

In the COU-AA-301 phase III study, 1195 patients who failed to one or two lines of chemotherapy were randomized to receive abiraterone 1000 mg daily plus prednisone 5mg bid versus placebo plus prednisone (68,69). Primary objective was met: median survival in patients treated with abiraterone was 15.8 months in comparison to 11.2 months in placebo treated patients ($p<0.0001$). Secondary objectives were time to PSA progression, radiographic progression-free survival (rPFS) and PSA response. Patients treated with abiraterone also obtained higher PSA response rate (29.5% vs 5.5%; $p<0.0001$).

In the predocetaxel setting, a phase III trial has evaluated the clinical benefit of AA vs prednisone in mildly symptomatic or asymptomatic chemo-naïve patients with progressive metastatic CRPC (70). In the COU-AA-302 study 1088 pts were randomized 1:1 to AA 1000 mg plus prednisone 5 mg bid versus placebo plus prednisone. Radiographic PFS was 16.5 and 8.3 months for AA and placebo, respectively (HR 0.53; 95% CI 0.45-0.62; $p < 0.0001$). In the final analysis median overall survival was significantly longer in the abiraterone acetate group than in the placebo group (34.7 months [95% CI 32·7–36·8] *vs* 30.3 months [28.7–33.3]; hazard ratio 0.81 [95% CI 0.70–0.93]; p=0.0033) (71).

Abiraterone has an excellent tolerance profile although it needs administration of prednisone in order to prevent the toxicity derived from the excess of mineralocorticoids (due to the CYP17 blockade). Most frequent grade 3-4 toxicities were edema and fuid retention (less than 3% severe), hypokalemia (<4%), hypertension (4%) and hypertransaminemia (3-5%) (68,70).

Enzalutamide (MDV3100) inhibits nuclear translocation of the androgen receptor, DNA binding, and coactivator recruitment (72). Compared to the currently available antiandrogen agents enzalutamide has a greater affinity for the receptor, induces tumor shrinkage in xenograft models and has no known agonistic effects.

In the double-blind AFFIRM trial, 1199 men with castration resistant prostate cancer after chemotherapy were randomly assigned (2:1 ratio), to receive oral enzalutamide at a dose of 160 mg per day or placebo. Unlike abiraterone corticoesteroids administration was optional in both arms. Enzalutamide was superior to placebo in the primary end point, overall survival (HR=0.63 [0.53, 0.75], $p<0.001$) (73). Enzalutamide was also superior to placebo in all secondary end points: reduction in PSA level by 50% or more

(54% vs. 2%, P<0.001), the soft-tissue response rate (29% vs. 4%, P<0.001), the quality of life response rate (43% vs. 18%, p<0.001), the time to PSA progression (8.3 vs. 3.0 months; HR 0.25; p<0.001), radiographic progression-free survival (8.3 vs. 2.9 months; HR 0.40; p<0.001), and the time to the first skeletal-related event (16.7 vs. 13.3 months; HR 0.69; P<0.001).

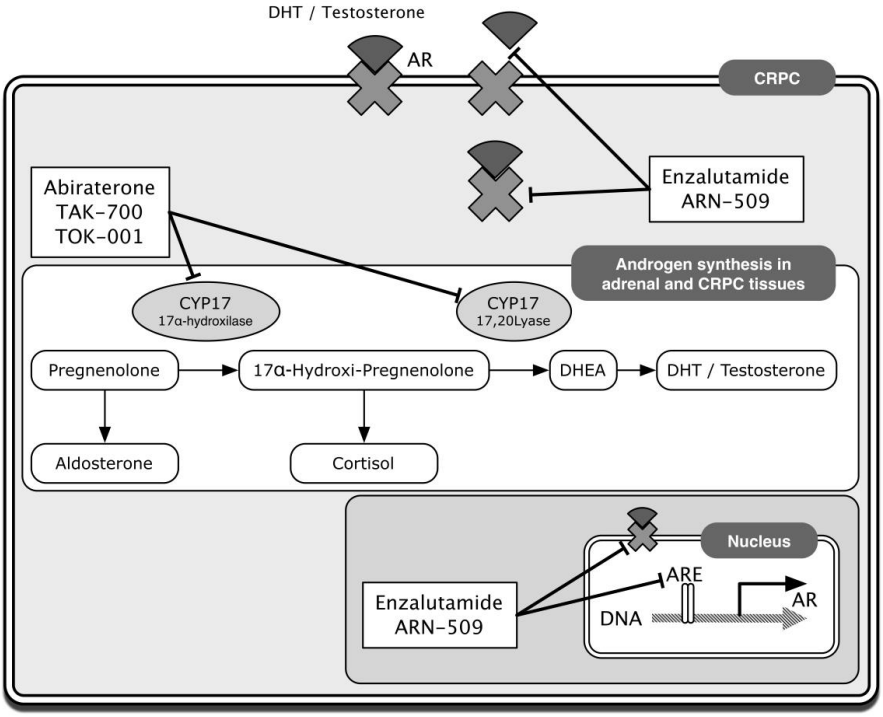

Figure 1.14. Interaction between testosterone and its receptor in the prostate cell. Adapted from González del Alba, León et al, "Targeted therapies for prostate cancer" (74).

Enzalutamide has a good toxicity profile, with fatigue (6% grade 3), hypertension (6.6%) and hot flushes (20%) as the main side effects. Cardiac events were observed in 6% of patients with enzalutamide versus 8% in the placebo arm. Seizures were reported in five patients (0.6%) receiving enzalutamide (73).

The PREVAIL phase III study, tested enzalutamide in chemotherapy-naive patients with mCRPC. A total of 1,717 men were randomized 1:1 to enzalutamide 160 mg/day or placebo (75). Median OS was 32.4 months (95% CI, 31.5–upper limit not yet reached [NYR]) in the enzalutamide arm vs 30.2 months (95% CI, 28–upper limit

NYR) in the placebo arm, with a 30% reduction in risk of death (OS: HR 0.70; 95% CI: 0.59-0.83; P< 0.0001). Median rPFS was not yet reached in the enzalutamide arm vs 3.9 months in the placebo arm, with an 81% reduction in risk of rPFS (HR 0.19; 95% CI: 0.15-0.23; P< 0.0001).

Chemotherapy.

Currently docetaxel based schemes are the standard treatment of patients with metastatic CRPC. In the SWOG 9916 trial, Petrylak et al compared docetaxel plus estramustine every 21 days and dexamethasone 20 mg x 3 doses versus mitoxantrone day 1 every 21 days with prednisone 5 mg every 12 hours (51). The median survival was 17.5 months and 15.6 months in the docetaxel and the mitoxantrone arm, respectively (p = 0.01). Docetaxel plus estramustine increased gastrointestinal toxicity (20% vs. 5%, p <0.001), metabolic disorders (6% vs. 1%, p <0.001), cardiotoxicity (15% vs 7%, p = 0.001), neuropathy (7% vs 2%, p = 0.001) and neutropenic fever (5% vs 2%, p = 0.01) without providing any benefit in pain control or quality of life.

In the TAX 327 trial 2 docetaxel schemes (weekly or every 3 weeks) were compared against the standard combination of mitoxantrone and prednisone (50). The primary endpoint was overall survival. The median survival was 16.5 months in the mitoxantrone group, 18.9 months in the group given docetaxel every three weeks and 17.3 months in the group given weekly docetaxel. As compared with the men in the mitoxantrone group, patients in the group given docetaxel every three weeks had a hazard ratio for death of 0.76 (95 % CI, 0.62 to 0.94; p=0.009) and those given weekly docetaxel had a hazard ratio for death of 0.91 (95 %CI, 0.75 to 1.11; p=0.36). Docetaxel given every 3 weeks caused 32% of grade 3-4 neutropenia, with only 2.7% of febrile neutropenia and no toxic deaths. Mitoxantrone and weekly docetaxel caused a 22% and 1.5% grade 3-4 neutropenia, respectively. There were no differences between the three schemes in non-haematological toxicity. Since the use of estramustine only caused more toxicity, docetaxel every 3 weeks plus prednisone was establihed as the standard chemotherapy regimen for the first line setting in metastatic CPRC (76).

Secondary objectives of the TAX 327 were pain response, PSA response, measurable response and quality of life. Docetaxel given every 3 weeks improved pain response when compared to mitoxantrone (35 vs 22%; p <0.01). The two docetaxel schemes (weekly or every three weeks) obtained higher response rate in the PSA compared to

mitoxantrone (45 vs 48 vs 32%, respectively).

In the TAX 327 and SWOG trials 99-16 docetaxel treatment was initiated without excluding patients according to the presence of symptoms or kind of progression. Asymptomatic patients have better overall survival, but it is unknown what is the consequence of delaying the start of cytotoxic therapy in these patients. Some men with asymptomatic metastatic CRPC can continue with no pain for long periods of time, while others have symptoms within a few weeks or months. In the first group we could delay the start of chemotherapy, while in the second group, with a survival rate around one year, it would be advisable not to delay treatment.

There is consensus that chemotherapy should not be delayed in symptomatic patients with visceral or bone progression, but in clinical practice often we consider these questions:

- Asymptomatic (or minimally symptomatic) patients do benefit from docetaxel use?
- All patients with asymptomatic CPRC should begin treatment with docetaxel?

To answer this question we should conduct a clinical trial in which asymptomatic or minimally symptomatic patients were randomized to early treatment or chemotherapy when symptoms worsen. In the absence of this phase III trial we need to find parameters that can help us in the decision making. Both in the original publication and updating of the TAX 327 trial can be observed that patients without pain at baseline showed an overall survival greater than those symptomatic (14.2 vs 21.3 months), but docetaxel is beneficial in both subgroups (50,77). In another analysis of the TAX 327 110 minimally symptomatic men were identified (78). Median survival in these patients were 28.4, 25.9 and 22 months for groups of docetaxel given every 3 weeks, docetaxel given weekly and mitoxantrone, respectively. Although these differences were not statistically significant due to the small sample size these data indicate that asymptomatic or minimally symptomatic patients benefit from the use of chemotherapy, and should not be excluded from treatment.

Cabazitaxel, a second generation taxane, is approved for men with mCRPC previously treated with a docetaxel-containing regimen, and it is recommended for patients with or without visceral metastases (79). The TROPIC trial randomized 755 men with progressive disease to receive cabazitaxel 25 mg/m^2 or mitoxantrone 12 mg/m^2, each with daily prednisone. Cabazitaxel achieved an improvement in OS up to 2.4 months (HR

0.72; p <0.0001). A higher toxic death rate was seen in men who received cabazitaxel (4.9 versus 1.9%). Febrile neutropenia was observed in 7.5% of cabazitaxel-treated men versus 1.3% of mitoxantrone-treated men. The incidences of severe diarrhea (6%), fatigue (5%), nausea/vomiting (2%), anemia (11%) and thrombocytopenia (4%) also were higher for cabazitaxel (80). These data suggest that cabazitaxel should be used with caution in patients with pre-existing bone marrow toxicities. A strict haematologic toxicity follow up, and prophylactic G-CSF could be recommended.

Inmunotherapy.

In the last years, immunotherapy has shown promising efficacy in the treatment of many solid tumors. Several different approaches with vaccines in prostate cancer have been evaluated in both the preclinical and clinical settings.

Sipuleucel-T, an autologous active cellular immunotherapy agent, was the first FDA-approved therapeutic cancer vaccine and other agents alone or in combination are being explored. In the IMPACT study, sipuleucel-T prolonged OS among asymptomatic or mildly symptomatic men with mCRPC, before or after docetaxel treatment, with a relative reduction of 22% in the risk of death as compared with the placebo group (HR: 0.78; 95% CI: 0.61-0.98; p=0.03) (81). This reduction represented a 4.1 months improvement in median survival (25.8 months vs. 21.7 months). The most common associated adverse events were chills (51%), fever (22%), fatigue (16%), nausea (14%) and headache (11%).

A phase 2 trial of PROSTVAC has shown an improvement of 8.5 months in overall survival (HR 0.56) (81). Accrual is ongoing to include 1200 patients in a phase III trial with three arms: ProstVac-VF, ProstVac-VF þ GM-CSF, or an empty vector control. Check-point inhibitors (ipilimumab, nivolumab) have shown impressive results in various types of tumors, but results in prostate cancer have been disappointing. Ipilimumab showed no survival improvement in a randomized phase 3 trial, but a subset analysis revealed a benefit in those patients without visceral metastases (82). A deeper understanding of the mechanisms of checkpoint inhibitor function and failure will lead to improvements in clinical trial design and subsequent better clinical results.

Bone targeted therapies.

Bone metastases occur in more than 80% of patients with advanced prostate cancer. Bone metastases in prostate cancer are related to osteoclast-mediated resorption.

Zoledronic acid was evaluated in a phase III trial compared with placebo (83,84). Median time to the first skeletal related event (SRE) was longer in patients treated with zoledronic acid (488 vs. 321 days; p = 0.01). Besides significantly more patients receiving placebo displayed SREs compared with those receiving zoledronic acid 4 mg (44% vs. 33%; difference:−11%; 95% CI: −20% to −2%; p = 0.021). Zoledronic acid requires monitoring for renal function, and is not recommended in patients with creatine clearance lower than 30 mL/min. Osteonecrosis of the jaw is a rare complication but it causes a serious deterioration in the quality of life.

Receptor Activator of Nuclear Factor Kappa B Ligand (RANKL) is involved in osteoclast function, formation and survival. Denosumab is a human monoclonal antibody directed against RANKL that inhibits osteoclast-mediated bone destruction. In a randomised, double blind study, subcutaneous denosumab was compared to intravenous zoledronic acid in 1904 patients with metastatic CRPC (85). The median time to first on-study SRE was 20.7 months (95% CI: 18.8–24.9) for denosumab compared with 17.1 months (95% CI: 15.0–19.4) for zoledronic acid (HR: 0.82; 95% CI: 0.71–0.95; p = 0.0002 for non-inferiority; p=0.008 for superiority). Adverse events were similar in both arms, but hypocalcemia and osteonecrosis of the jaw occurred more frequently in the denosumab group. Denosumab had no effect on renal function and there is no need for renal monitoring.

Radium is a calcium mimetic bone seeker alpha-particles emitter able to replace areas of increased bone turnover. Radium 223 (223Ra) has been introduced recently, representing the first alpha-emitter drug available for the treatment of patients with CRPC, with symptomatic bone metastases and no known visceral metastases. The ALSYMPCA trial was an international, randomized, double-blind, phase 3 study conducted in men with symptomatic mCRPC comparing 223Ra with placebo (86). The trial enrolled patients progressing on, not eligible for, or refusing prior docetaxel-based chemotherapy.

Radium 223 significantly improved OS compared with placebo (14.9 months vs. 11.3 months, respectively; HR: 0.70; 95% CI: 0.58–0.83; p<0.001). Time to first SRE was significantly prolonged (median 15.6 months versus 9.8 months, respectively; HR = 0.66; 95% CI, 0.52-0.83; p <0.001).

Table 1.5. Selected Ongoing clinical trials of targeted therapy for patients with metastatic CPRC.

Ag Agent	Phase	n	Setting	Comparison Treatment	Clinical Trials.gov Identifier
Chaperone protein					
Custirsen	III	1023	chemotherapy naive mCRPC	Custirsen+Docetaxel and Prednisone Docetaxel/prednisone	NCT01188187 SINERGY
Custirsen	III	630	mCRPC after docetaxel failure	Custirsen+Cabazitaxel and Prednisone Cabazitaxel/Prednisone	NCT01578655 AFFINITY (recruiting)
OGX-427	II Rand	74	mCRPC PSA progression with Abiraterone	OGX-427+Abiraterone and Prednisone Abiraterone/Prednisone	NCT 01681433 (recruiting)
OGX-427	II Rand	72	mCRPC in progression without prior chemotherapy	OGX-427+Prednisone Prednisone 5 mg BID	NCT01120470
PI3K/PTEN/mTOR					
BEZ235	Ib/II	74	Asymptomatic or minimally symptomatic mCRPC	BEZ235+Abiraterone and Prednisone	NCT01717898 (finish)
GDC-0068/GDC-0980	Ib/II	262	mCRPC previously treated with docetaxel	GDC-0068+Abiraterone and Prednisone Abiraterone/Prednisone	NCT01485861 (recruiting)
Everolimus	I/II	60	mCRPC	Everolimus+docetaxel/ Prednisone	NCT00459186 (completed)
IGFR-1					
Cixutumumab (IMC-A12)	II	41	Asymptomatic mCPRC Chemo naive	Cixutumumab (single arm)	NCT 00520481 (completed)
Ramucirumab (IMC-1121B)	II Rand	133	mCRPC after docetaxel failure	Cixutumumab+Mitoxantrone /Prednisone Ramucirumab+Mitoxantrone and Prednisone	NCT00683475 (completed)
Figitumumab (CP-751,871)	II	120	mCRPC Chemotherapy -naïve or docetaxel-refractory	Figitumumab +Docetaxel/Prednisone (single arm)	NCT00313781 (completed)
PARP inhibitor					
Olaparib (AZD2281)	II	50	mCPRC postdocetaxel	Single arm	NCT01682772 TOPARP
Veliparib (ABT-888)	II Rand	148	mCRPC	Veliparib+Abiraterone and Prednisone Abiraterone/Prednisone	NCT01576172 (recruiting)
Immunomodulator					
Tasquinimob	III	1200	asymptomatic or mildly symptomatic mCRPC	Tasquinimob Placebo	NCT 01234311

Targeted therapies.

Besides AR mediated pathways, several alternative signaling pathways may also be involved in the disease progression of prostate cancer. Prostate cancers are often characterized by abnormalities in a variety of growth factor signaling pathways that control cell cycle and apoptosis. As these pathways are being understood, new therapeutic drugs are being developed directed against related targets. The review of each of these agents is beyond the scope of this thesis. Table 1.5 summarizes several agents at different stages of development in the treatment of mCPRC.

Several of these agents have demonstrated promising activity in early stages, not confirmed later in randomized trials. Again, the absence of biomarkers to select the most appropriate drug for each patient is a barrier that must be overcome to improve current results.

3. Evaluation of response and progression criteria in metastatic castration resistant prostate cancer.

A significant proportion of patients diagnosed with prostate cancer will progress after the initial treatment, which will inevitably lead to the appearance of metastasis and subsequent resistance to androgen deprivation therapy. Traditionally, the assessment of the response to the therapeutic agents of this disease has been particularly complicated due to the absence of easily measurable lesions, the prevalence of bone spreading and the relatively prolonged natural history in a significant number of patients.

In 1999, the Prostate-Specific Antigen Working Group (PCWG1) defined the eligibility criteria and treatment benefit in clinical trials and proposed specific criteria for the use of prostatic specific antigen (87). The Response Evaluation Criteria in Solid Tumours (RECIST) and subsequently the modified RECIST criteria 1.1 tried to establish standards for the response evaluation in solid tumours, although they are often difficult to apply alone in the case of prostate cancer (88,89). Subsequently, the Prostate Cancer Clinical Trials Working Group (PCWG2) established a number of progression criteria to address the inclusion of patients in clinical trials and the evaluation of effectiveness of new treatments (64). These criteria, with some modifications, currently remain in force. However, controversies over the methods and more appropriate criteria for the best evaluation of castration-resistant prostate cancer progression remain.

As previously mentioned following the establishment of the docetaxel-prednisone regimen as the first-line standard of care in patients with metastatic CRPC, several agents have been shown to increase overall survival in patients participating in phase III trials (50)((68-71,73,75,80,86). Therefore, given the difficulty in assessing the disease, as well as the availability of new treatments that affect its survival and the nature of the various agents involved, the progression criteria to be used in clinical trials and in the clinical practice for all the phases of mCRPC need to be defined. Given the limitations in the assessment of response by PSA and imaging methods, the use of CTC in this scenario can be really useful.

Progression criteria in CRPC.

The definition of castration resistance in patients with prostate cancer involves biochemical (PSA) and/or clinical progression under adequate castration conditions. Therefore, CRPC requires two essential conditions: (1) adequate castration defined as serum testosterone levels < 50 ng/dL, and (2) evidence of progression (90).

As mentioned above, the definition of progressive disease in patien ts with mCRPC has been the subject of multiple considerations and continues to be an unresolved issue. In 2008, the PCWG2 criteria established a consensus for the progression criteria assessment and the disease status in these patients (64). Modification of the old criteria should allow the selection of the most suitable patients for clinical trials and the evaluation of the effectiveness of new treatments.

One of the most important concepts introduced by the PCWG2 is the definition of five patterns of spread, each one with a different natural history and prognosis. The OS varies between 9 to 48 months, depending on pattern of tumour spread, which include the following: (1) locally progressing tumors and no metastatic disease, (2) biochemical progression (rising PSA-castrate) and no detectable metastatic disease (median OS 4 years), (3) nodal spread and no evident bone or visceral disease (median OS between 18 and 24 months), (4) bone disease with or without nodal disease and no evident visceral spread (median OS 18 months), and (5) visceral metastases (median OS between 9 and 16 months).

PSA progression is defined as three consecutive increments in the PSA value, at a minimum interval of one week, resulting in two increments of, at least, 50% of the nadir value, provided the PSA value is > 2 ng/mL. Radiological progression is defined as the

appearance of two or more new lesions in bone scintigraphy (conclusive proof) or progression by RECIST 1.1 criteria (in the case of soft tissue lesions). Finally, clinical progression is defined as pain progression or development of skeletal events (e.g., pathological fracture, medullar compression, palliative radiation or bone surgery).

In addition to defining the patterns of spread, the PCWG2 divided the aims of treatment of patients with mCRPC into the following two categories: (1) control, alleviate or eliminate the symptoms of the disease once treatment is started and (2) prevent or delay symptoms of the tumour in the future. For this reason, it´s recommended focusing on time-to-event objectives, advising to document pain at the start of treatment and every 3-4 weeks, monitor symptoms and quality of life and ignore early changes in pain ($\leq$ 12 weeks) in the absence of progression, and confirm response or progression of symptoms after $\geq$ 3 weeks. Monitoring symptoms, such as pain, is an important therapeutic goal and should be considered an independent objective due to the poor correlation between PSA response and pain response, since the presence of pain constitutes an adverse prognostic factor for survival (78).

What is the role of PSA doubling time (PSA-DT)?

In a retrospective study, Oudard *et al.* evaluated the usefulness of the PSA-DT prior to chemotherapy as a subrogated marker of survival in CRPC. They observed that the median survival was significantly lower if the PSA-DT was less than 45 days compared to PSA-DT values above 45 days (16.5 months vs. 26.4 months, respectively) (91).

Various published studies confirmed that the PSA response rate to chemotherapy in the CRPC is correlated with survival and is an acceptable measure of the potential benefit to the patient (92,93) . Hussain *et al.* reported results of a retrospective study of 1015 patients, in which PSA progression, defined by PCWG 1 and 2 criteria, was correlated with survival, both in the hormone-sensitivity and in the CRPC phase (94). Therefore, PSA progression is a powerful predictor of survival, and thus can be considered a good primary endpoint for phase II trials. For this reason, the evaluation of PSA kinetics is currently recommended in order to assist in the decision-making, especially in those cases with no clear indication of when to start chemotherapy.

When should treatment start in patients with mCRPC?

Among the treatment options for patients with mCRPC who experienced progression despite an androgen deprivation therapy (surgical or chemical castration with luteinizing

hormone-releasing hormone analogues plus an anti-androgen agent), there is a wide variety of "classic" second-line hormonal approaches, including discontinuation of the anti-androgen therapy, high doses anti-androgen, adrenal inhibition with ketoconazole (although this has been withdrawn due to the risk of hepatotoxicity) or the use of corticosteroids (95-97) .

According to the recommendations published by the Spanish Oncology Genitourinary Group (SOGUG), classic second-line hormonal approaches should be reserved for patients with a slow progression of the disease, as well as those who are asymptomatic or mildly symptomatic and those in which chemotherapy is contraindicated (67,98) . However, none of these classic second-line hormonal therapies had an impact on survival, obtaining only short-term responses (11).

When should treatment with docetaxel, abiraterone or enzalutamide start?

The final results of the COU-AA-302 study confirm the anticipated clinical efficacy in the preliminary analysis (71). The established criteria to determine progression were clinical (e.g., pain, need for radiotherapy or chemotherapy, or worsening of the performance status) and radiological (i.e., the PCWG2 criteria adapted to the trial). Both COU-AA-302 and PREVAIL included patients with progressive, asymptomatic or minimally symptomatic mCRPC (75). The patients included had PSA progression according to the PCWG2 criteria or radiological progression. According to the results of these trials, abiraterone-prednisone and enzalutamide can be considered the alternatives of choice in this group of patients.

On the other hand, patients with symptomatic mCRPC or with objective progression of visceral metastases, as well as those at a greater risk of a rapid progression, should be considered candidates to receive chemotherapy. In patients with these conditions in which an increased risk of hematologic toxicity is expected or with an initial worsening of the performance status, a treatment with docetaxel administered weekly might be appropriate, due to its lower expected hematologic toxicity.

The asymptomatic patient is defined in pre-chemotherapy studies as one that does not require the use of opiates nor radiotherapy for pain control and, in general, presents increased PSA as the only progression criterion. In the TAX 327 study, patients without pain at the study start had a greater OS than symptomatic patients (21.3 vs. 14.2 months, respectively), although the benefit of docetaxel was maintained in both subgroups. For

this reason, treatment with docetaxel for these patients should be considered based on their characteristics, especially if they are not candidates to receive abiraterone or enzalutamide (78).

As mentioned above, asymptomatic patients have better OS, but the potential consequences of delaying the onset of treatment in these patients should be taken into account. Some patients with mCRPC may be asymptomatic for long periods of time, while others will have symptoms in a few weeks or months. Given the short OS in the latter group (approximately one year), delaying treatment initiation would not be recommended, especially considering that there are several drugs that have shown benefit in phase III trials. Therefore, patients with mCRPC should always be closely monitored, including regular clinical assessments and frequent PSA measurements.

Taking into account all the variables and the available evidence already described, abiraterone plus prednisone or enzalutamide could be the first treatment for patients with minimally symptomatic mCRPC without visceral metastasis. Similarly, it seems advisable to give chemotherapy with docetaxel as the first therapy especially to the group of patients with symptomatic mCRPC with visceral metastases.

Due to the toxicity profile of chemotherapy, one of the controversial issues in mCRPC is the optimal time to start treatment. In general, the evaluation of clinical and biological parameters, such as the PSA-DT, is considered when selecting patients. In this sense, a multidisciplinary team should take the decision, and the potential benefit and side effects of the treatment proposed should be discussed with each patient.

What information can be obtained from prognostic nomograms?

Several nomograms have been published based on the prognostic variables analyzed in groups of patients with CRPC treated with first-line chemotherapy (99-101). For example, the nomogram described by Armstrong *et al.* incorporated PSA kinetics to predict survival at 1, 2 and 5 years in patients with CRPC treated with docetaxel in the TAX 327 trial and included new independent clinical factors (92). Thus, it has become a useful tool for stratifying patients who might be candidates for inclusion in clinical trials. However, in the routine clinical practice, the use of nomograms is not generalized; thus, their usefulness would be limited with respect to deciding the best timing to start chemotherapy.

Criteria to start a second-line treatment (post-docetaxel).

Apart from meeting several criteria to define disease progression, and with various effective drugs in the postdocetaxel setting of CRPC, it is important to know the prognostic factors of such a progression. Armstrong *et al.* identified prognostic variables for OS pre- and post-chemotherapy, useful in deciding when to start treatment (Table 1.6). Given their impact on survival, it is important to know the percentages of patients included in clinical trials with any of these clinical manifestations, which are shown in Table 1.7.

Table 1.6. Multivariable model for overall survival after disease progression used for the construction of the nomogram (modified from Armstrong *et al*. (102)).

Prognostic factor	HR	CI 95%	*P*-value
Pre-chemotherapy variables			
Liver metastases	1.48	0.94-2.30	0.089
Significant pain	1.31	1.08-1.56	0.005
>2 metastatic foci	1.71	1.23-2.25	0.001
Worst performance status (KPS ≤ 70)	1.39	0.97-1.92	0.063
Time from diagnosis (y)	0.95	0.93-0.99	0.004
Presence of anaemia (Hb < 13)	1.30	1.05-1.58	0.012
Alkaline phosphatase, IU/dL (<200 vs. 200-1000)	1.26	1.02-1.55	0.033
Alkaline phosphatase IU/dL (<200 vs. > 1000)	1.55	1.11-2.21	0.012
Post-chemotherapy variables			
Duration of first-line chemotherapy (months)	0.95	0.91-0.99	0.022
Number of progression factors (2 vs. 1)	1.26	0.95-1.60	0.073
Number of progression factors (3 vs. 1)	2.64	1.86-3.75	<0.0001
Progression during chemotherapy (yes/no)	1.72	1.24-2.32	0.001

Note: The corrected c-index for confidence intervals is 0.7049, n=640.

Abbreviations: HR, hazard ratio; CI, confidence interval; KPS, Karnofsky Performance Status; Hb, haemoglobin.

There is no absolute consensus on the criteria to be used in deciding when to start treatment after progression on docetaxel. However, based on the inclusion criteria of published studies, patients with an Eastern Cooperative Oncology Group (ECOG) performance status ≤ 2, with clear progression, with or without the presence of symptoms associated with cancer and without relevant co-morbidities should be considered candidates for treatment. As a general consideration we recommend that these data should be evaluated by the medical oncologist and discussed with the patient, to take a consensus decision considering the benefits and toxicity associated with treatment.

Table 1.7. Clinical manifestations of CRPC progression (%).

Manifestation	MSKCC (N = 124)	SWOG9916 (51) (N= 770)	TAX 327 (88) (N=1,106)
Increased PSA	94	90	87
Bone	84	88	93
Significant pain	35	36	36
Soft tissue lesions			
Lung /liver	16	19	22
Lymph nodes	24	24	18
Prostate/prostate bed	2	Not specified	Not specified

Abbreviations: MSKCC, Memorial Sloan-Kettering Cancer Center; SWOG, Southwest Oncology Group; PSA, prostate-specific antigen.

4. Clinical applications of circulating tumors cells in patients with prostate cancer.

Currently, histopathological analysis (Gleason score) and PSA levels are key determinants of therapeutic decision. However, PSA analysis have some weaknesses as a biomarker, because it is also increased in benign prostatic hyperplasia, their levels could be similar in indolent and aggressive cancers and often fails to indicate accurately the patient`s response to a given treatment. In addition, the histopathological analysis is not enough to predict the disease evolution (103). Therefore, the clinical application of new surrogate markers will provide the opportunity for improving patient management and the therapeutic selection and monitoring.

For its clinical use, a novel biomarker should provide relevant information to the clinicians in a cost-effective way. Therefore, new biomarkers need to improve the standards or at least improve their accuracy. The ideal biomarker should be determined through a blood test providing evidence about a patient's outcomes (prognosis marker) or predicting the likelihood of response/benefit to a specific therapy (predictive marker) (104).

During the process of haematogenous spread in prostate cancer tumor cells travel through the blood vessels and, after extravasation, colonize distant target organs, typically the bone. As circulating tumor cells (CTCs) are an intermediate between the primary tumor and metastasis, they are candidates to act as a surrogate markers measurable in blood (105). The quantification of these CTCs has experienced a rapid technological development in last years, allowing the accumulation of important data to establish the potential clinical value of CTCs as early detection, diagnostic, prognostic, predictive, surrogate, stratification, and pharmaco-dynamic biomarkers in different carcinomas.

CTCs are widely recognized as a biomarker in PCa. Numerous studies have demonstrated the association between CTCs baseline levels and clinical outcomes in metastatic patients (106-108). In addition, drops in CTCs levels within the therapy has been associated with higher overall survival, similar to the benefit correlated to a substantial PSA decrease or radiographic response (107,109,110). Besides, changes in CTCs levels usually precede PSA fluctuation being their monitoring of even greater value when changes in PSA or bone disease are difficult to evaluate (108). Despite of

all the promising data regarding the value of CTCs as a disease marker in prostate cancer, their clinical utility should be carefully interpreted.

Current and new approaches for CTCs detection: application in prostate cancer.

CTCs occur at very low frequency in the bloodstream, generally estimated at 1 CTC per million of leukocytes. Because of the low concentration of CTCs in blood, extremely sensitive and specific strategies are required to process the blood samples in a short period of time. CTCs enrichment methods are based on physical or biological cell properties such as size or specific marker expression (Table 1.8). Immunocytochemical enrichment is the most employed strategy to isolate CTCs from blood cells. Affinity techniques normally use antibodies which recognize antigens expressed by CTCs but not by blood cells. The antigen mostly used is EpCAM, an epithelial marker overexpressed in some carcinomas (111,112).

Table 1.8. CTCs enumeration platforms for prostate cancer.

Assay	Enrichment	Detection
Cellsearch system	Immunocapture (EpCAM)	IF for CK, CD45, and DAPI
MagSweeper	Immunocapture (EpCAM, CD45)	PCR for PSA, KLK3, TMPRSS2, CD45
EPISOT assay	Immunocapture (CD45, CXCR4)	Secretion of proteins; CK19, MUC1, PSA
ISET	Cell size	ICC for CK
ApoStreamTM	Dielectrophoretic device	ICC for EpCAM and CK
CTC Membrane Microfliter	Cell size	IF for CK
DEPArray	Microfluidics	Image-based selection
Nanodetector (GILUPI)	Immunoisolation (EpCAM)	IF ofr CK, EpCAM, CD45
Ficoll-Paque	Cell Density	ICC for CK, PSA PCR
Vita-Assay (Functional Collagen Adhesion Matrix)	Marker independent isolation	ICC or flow cytometry (EpCAM, CK, CD44, CD34, CD45, Vimentin)
GEDI microfluidic device	Microfluidic/Immunocapture (PSMA)	ICC for CD45, PSMA, EpCAM
RosetteSep	Deplection of CD45	PCR, ICC, cell culturing
CTC-iChip	CD15, CD45 +/- EpCAM	Immunofluorescence, cytopathologic, FISH
Magsweeper	Immunoisolation (EpCAM)	PSA, KLK3, TMPRSS2, CD45

However, CTCs undergo changes in their epithelial signature during the metastatic process interfering with the use of EpCAM as an universal marker (113,114) . Therefore, big efforts are focused on characterization and identification of additional markers able to distinguish CTCs from their counterparts in blood. Moreover, it is important to distinguish viable from apoptotic CTCs and to detect and profile the most relevant metastasis-initiating CTCs.

Other methods for CTC enrichment or isolation are based on size, density, electric charges, or deformability. In this sense, several platforms using size as the isolation method to detect CTCs from blood were reported in recent years (115-117). An example of theses commercial available devices is ISET or ScreenCell® (115,116,118,119). The handicap of these systems is that they provide low CTCs purity requiring in most cases further enrichment, and the fact that leucocytes could overlap in size with CTCs. An alternative approach is enrichment of cells by their density, such Ficoll–Hypaque method (120). This principle allows a marker-independent cell selection but leads to a high loss of tumor cells conducting to false-negative results in clinical samples.

Microfluidics have been demonstrated to be valuable platforms for CTCs analyses that can be integrated to other processing steps to fully automate sample processing. These systems could combine both physical and biological strategies. Microfluidic devices based on affinity selection typically show higher purities compared to size-based selection but at the expense of throughput (112,121,122). For example, “CTC-chip” isolates viable CTCs by affinity to anti-EpCAM-coated microspots under controlled laminar flow conditions. This approach demonstrated higher sensitivity, selectivity and yield compared with techniques based only on immunomagnetic beads in prostate cancer patients (112,123) .

CellSearch (Veridex) is the only technology approved by the US Food and Drug Administration (FDA) for the CTCs quantification in metastatic breast, prostate and colon cancer. This technology uses magnetic beads coated with an anti-EpCAM antibody for the CTCs isolation; the identification is mainly based on cytokeratin-positive expression. Although CellSearch is an accepted platform with high value for cancer prognosis and monitoring, its limitation to some cancer types reinforce the need of more effective technologies for the CTCs analysis (124,125) .

Recently, many new technologies have been presented for CTCs detection and molecular profiling but the clinical utility of most of them have to be demonstrated. There is a big interest in developing microdevices that can process smaller volumes of blood decreasing the assay time and the cost. Opposite, some technologies are now directed to analyse larger blood volumes, particularly in early-stage tumors where CTCs presence is even more unfrequently (126). Overall, efforts are directed to develop devices for live cancer cell detection with single cell sensitivity, high selectivity and reproducibility, easy fabrication and low cost. A strict clinical validation of the new devices is now required before their introduction into the management of cancer patients.

Application in prostate cancer.

Given the difficulty to acquire biopsies from patients with only bone metastasis and the value of providing new biomarkers (which improve PSA information) that could be used as surrogates for survival in clinical trials, prostate cancer represents the ideal disease for CTC research and clinical development. Importantly, prostate cancer cells display tissue-specific antigens, such as PSA and PSMA usually absent in non-epithelial cells. In addition, prostate cancer CTCs express epithelial markers such as EpCAM and several cytokeratins (8-9-19) in a higher degree that CTCs originating from other tumors even if the cells are heterogeneous and some of them display with EMT-like characteristics (127).

Chronologically the first approaches for CTC detection in prostate cancer were based on RT-PCR analysis to detect prostate-specific or epithelial specific markers in non-pre enriched blood samples (128,129). The results of these studies showed the possibility to distinguish patients from controls based on the levels of mRNA, but were often unable to show a direct prognostic relationship. Potential limitations of this approach are the low sensitive due to white blood cells contamination, the variability in the number of copies of a given mRNA between cancer cells and the use of heterogeneous inclusion criteria and small size numbers in confirmatory trials.

The next step for CTC research development in prostate cancer was the use of complex methods with a selection step previous the cell identification. Using the CellSearch technology, as we commented before, tumour cells are first positively selected based on the EpCAM expression and then the identification depends on morphology

characteristics and the presence of cytokeratins expression (125).

This has permitted to find specific mutations such as the TMPRSS2-ERG translocation, the loss of PTEN and overexpression of markers such as the androgen receptor, also found in primary tumors, confirming that these cells are not only epithelial but originated in the tumor (130-132). Interestingly, there are also subtle differences favoring a more mesenchymal-like phenotype in the CTCs, perhaps reflecting that only a subset of cancer cells is able to intravasate and become a CTC (127).

Although EpCAM is the most widely used marker for positive selection of CTCs, other prostate cancer markers such as PSA and PSMA have been used with promissory results. Negative selection methods and methods that use physical properties offer the potential advantage of capturing cells that have lost the expression of epithelial and cancer specific markers, and have also showed some efficacy in prostate cancer (133-135).

In last years, new CTC methodologies in prostate cancer have focused on offering a high sensitivity and the potential to conduct molecular characterization studies. Some of these technologies combine antibody specific binding and physic properties in microfluidic systems that may offer higher sensitivity and also more purity for cells with lower EpCAM expression (136,137) .

Clinical development of CTCs as a biomarker in prostate cancer.

Requirements for biomarker validation.

The first effort to standardize the requirements for the clinical validation of biomarkers was the development of common guidelines for reporting the results of studies with tumor markers, REMARK consensus (reporting recommendations for tumor MARKER prognostic studies) following the recommendations of the NC-EORTC (First International Meeting on Cancer Diagnostics in 2001) (138).

In 2004 the FDA Critical Path Iniciative identified the lack of adequate biomarkers as an obstacle for drug development and promoted the foundation of a ACCR-FDA-NCI backed Cancer Biomarkers Collaborative (CBC), a new consortium founded to "accelerate the translation of cancer therapeutics into the clinic by shaping the processes for the effective development of validated biomarkers and their use in clinical trials". In 2010 the CBC committed to 27 recommendations for the clinical validation of new molecular biomarkers (139). These recommendations focus on eight different areas to

improve biomarker development: biospecimens, analytic performance, standardization and harmonization, bioinformatics, collaboration and data sharing, stakeholder education and communication, regulatory issues, and science policy.

More clear is the proposal of the Evaluation of Genomic Applications in Practice and Prevention (EGAPP) working group, which consists in three successive requirements: *analytic validity*, *clinical validity*, and *clinical utility* (140). While analytical validity refers to the reproducibility, accuracy and reliability of a test, clinical validity entails that the test can identify the phenotype, disease or the subgroup of patients of interest. Therefore, clinical validity encompasses analytical validity but has also to take into account the specificity and the disease prevalence to calculate the positive and negative predictive value. Finally, clinical utility requires that the use of a test provide with an added benefit for patient management-making.

Although the level of evidence for each of these components can be evaluated objectively based on pre-specified benchmarks it is important to note that clinical utility requires both clinical and analytical validity (141). In this sense, a test validated in a randomized controlled trial has also to perform reliably in external quality assessments in order to be useful for clinical use.

Finally, a recent document redacted by the European Group on Tumor Markers, established a four phase model for biomarker validation, analogous to the pathway for therapeutic trials in oncology (104). This pathway clarifies the strategies and objectives in each step of the process and provides several recommendations to guide trial design for the validation of a tumor biomarker.

Obstacles for the clinical use of CTCs.

Analytical validation.

From an analytical point of view a validated method has to show high reproducibility of results in different measures from the same patient, but this is not always possible for CTCs enumeration. One of the main sources of variation is the low concentration of CTCs in blood, up to 30-40% of metastatic patients and more of 90% of patients with localized disease fail to have more than 5 CTCs (142). Thus, differences of one CTC between different measures could involve the classification of patients in the group of good *vs*. poor prognosis. A potential way to improve CTC yields, even without

improving sensitivity, would be the use of larger samples of blood, increasing the number of expected CTCs.

The sensitivity of the different methods is normally tested using culture tumour cells mixed with blood. It is known that patient derived CTCs are smaller, more heterogeneous and may express different markers that cancer cells in culture, and probably a better surrogate -such as cells from primary tumors- should be employed (143). In this sense, the absence of epithelial markers in CTC samples due to EMT impairs recovery rate in patients with a potential poorer prognosis.

Another critical source of error is the variability in the analytical method chosen. In this regard, data with the CellSearch method in 14 different laboratories showed that, although the inter-instrument and inter-assay reproducibility of the assay was high, the inter-laboratory reproducibility was low, due to inter-observer variation (35). This finding has been reproduced in other studies (144).

Recent studies comparing different methodologies in paired samples from the same patient have shown that microfluidic systems and other methods that do not depend on EpCAM expression can isolate a higher number of CTC compared with CellSearch, even in samples that are negative for the CTC counting using the reference method. It is important to conduct additional studies focusing in quality control and benchmarking the performance and reliability of this methods.

Clinical validation.

Although most methodologies for CTC isolation and quantification require a prospective collection of samples, many studies have been designed using cohorts without a clinically significant pre-specified primary endpoint or a preplanned sample size calculation. As a result, many series may be underpowered (1-B<80) to verify a clinically relevant prognostic effect or confused by too permissive inclusion criteria (145). Other potential issue is the fact that although CTC isolation techniques are highly specific (over 98% for most techniques), sensitivity is still poor. Regarding this issue, CTC fragments have demonstrated to have prognostic value even in patients with no conventional CTC, suggesting a critical impact of sensitivity for the clinical validation of new technologies (146,147) .

Even considering these limitations, the hazard ratio related with high count of CTC presented a proper magnitude to be clinically significant, mainly in prostate cancer patients.

Actual evidences supporting the clinical use of CTCs in prostate cancer.

Localized disease.

Some investigators have evaluated the number of CTC in patients with localized prostate cancer. In a study performed in 37 patients (only 8 had non-metastatic disease) the authors related a cut-off of 5 or more CTCs/7.5 ml with poorer survival (148). The main limitations of this study were the small number of patients with localized disease, the wide range of PSA (from 0.2 to 22.6 ng/mL) and the absence of pathologic confirmation of staging.

Gewanter *et al.* detected circulating prostate cancer cells using PSA levels analysis by RT-PCR in the serum of 161 patients with localized Prostate cancer treated with radiotherapy (149). The median follow-up was 29 months. The pretreatment RT-PCR result was not predictive of biochemical relapse-free survival or clinical disease-free survival. Only in 25 patients with T3-4 prostate cancer the pretreatment negative RT-PCR was associated with better outcomes.

Berg and collaborators analyzed retrospectively the impact of disseminated tumor cell (DTCs) in biopsy material from bone marrow by immunohistochemical techniques in 272 patients with prostate cancer (cT1-4 pN0M0) treated with radiotherapy (150). The presence of DTCs in bone marrow at diagnosis was associated with the histological differentiation of the primary tumor and an increased risk of developing distant metastases after radiotherapy.

Advanced disease.

In 2008 de Bono and colleagues conducted the IMMC38 study in 231 metastatic CRPC patients treated with chemotherapy (107). Patients with more than 5 CTC/7.5mL blood were associated with worse overall survival (11.5 *v* 21.7 months; HR: 3.3; $P < .001$), distinguishing patients into favorable and unfavorable groups. CTC count showed even greater prognostic value than PSA levels. This study led to the approval by the FDA of the quantification system CellSearch CTC for advanced prostate cancer. A follow-up study of the same cohort analyzed only those patients receiving first-line therapy and showed that absolute CTC count and changes in CTC count measured as continuous variables were prognostic for survival in this group (108). In another study conducted in 162 mCRPC patients who received docetaxel, CTC levels at baseline (cut-off 5 cells /

7.5 ml) and at 2-5 weeks (< or ≥ 5 cells/7.5 mL) correlated with survival, while the decline in PSA (30 or 50%) did not (151).

The prognostic value of ≥ 5 CTCs/7.5 mL of blood was confirmed in the randomized COUAA-301 phase III study of abiraterone acetate in docetaxel-refractory mCRPC (152). In this study, CTCs were enumerated at baseline and during the first three cycles. CTC conversion from ≥ 5 CTCs to < 5 CTCs, along with changes in serum LDH, was strongly predictive for OS. Similar results were recently observed in a phase III study using Docetaxel with or without Lenalidomide (153). Blood samples for CTC analysis were collected from 208 patients: 105 received docetaxel plus lenalidomide (DL) and 103 received docetaxel (D). Baseline CTC counts were <5 cells/7.5 ml in 87 pts and ≥5 cells/7.5 ml in 121 pts. Overall, 2-year OS was lower in patients with baseline CTC ≥5 in both arms (DL, HR 3.63, p=0.0044; D, HR 3.41, p=0.0459). An increase in CTC between baseline count and cycle 4 was associated with significantly shorter OS (HR 5.24; p=0.0251).

The phase III SWOG 0421 trial compared the effectiveness of docetaxel plus atrasentan, versus docetaxel alone (154). The study failed to meet its co-primary end-points of improved OS and progression-free survival with the addition of atrasentan. As part of the study, CTCs were enumerated at baseline and 21 days after the first dose of treatment. The authors showed that baseline CTC counts were correlated with recognized prognostic markers, including PSA, alkaline phosphatase, hemoglobin, liver disease and bone pain. Unfortunately, relationship with LDH was not assessed.

Nowadays, there are no robust data supporting the surrogacy of CTCs enumeration and patient´s outcome using different methods than CellSearch, in spite of the promising results in terms of sensitivity demonstrated by the new technologies.

Challenges to improve CTC use in clinical and preclinical scenarios.

To introduce the use of CTC and derived biomarkers in the day-to-day management of prostate cancer, we can not forget the necessity to prove the cost-efficient of their analysis to be reimbursed in many health systems. It is important to remark that although having the FDA approval since 2006, CellSearch is still considered investigational by many insurance providers, and has not been evaluated by agencies such as NICE or others. To improve the cost-effectiveness ratio of the CTC analysis the cost of the CTC enumeration should be reduced and the molecular characterization

upgraded to increase the value of doing a CTC enumeration in terms of quality-adjusted-years gained.

There are clinical scenarios where the prognostic information provided by a CTC analysis could change the clinical practice. For example, when clinicians have to determine if a patient will survive enough to derive benefit from an immunotherapy that needs three to six months to be effective, or weather a surgical palliation of a spinal cord compression is guaranteed. In these cases clinicians use prognostic estimators based in clinical and analytic parameters, but there is evidence that CTCs improve the predictive value, specially to decide phase I enrolment (155).

Even more important is the relationship between CTC increases and decreases within the course of the disease. The CTC levels could help oncologist to distinguish an early PSA and radiographic flare from a real progression, or when a clinical deterioration without PSA changes is observed suggesting switch of the tumour to a neuroendocrine pathology. This is supported by a high correlation found between CTCs changes and survival in several phase III trials (107,108,154). In fact PSA changes after treatment were not always prognostic alone in these studies and should not be used to make treatment changes.

Currently, there are clinical studies exploring the value of the CTC count to be used as decision guidelines: NCT01710605 in breast cancer or NCT01640444 and NCT01640405 in colorectal cancer. In these trials the underlining hypothesis is that patients with high CTCs levels have a more aggressive disease and need to be treated with less conservative therapies. Although it has not been proved that the adverse prognosis of having more CTCs is affected by a more aggressive treatment, patients who change from a high number of CTC to a low number of CTCs after treatment, live as long as patients with low basal number of CTCs (107).

One interesting clinical application of these differences in tumor biology between the high and the low CTC group is the evaluation of new treatments, especially for strategies that may be associated with more toxicity only in the high CTC subgroup. With this approach the potential toxicities could be minimized, the number of patients to show a benefit would be reduced and the patients could be treated according to their underlining biological profile. Examples of studies limited to this CTC high subpopulation are the NCT01499043, where only patients with more than 10 CTC

detected by basal CellSearch analysis are included in the study and treated with the multi-targeted tyrosine kinase inhibitor PLX3397. A 10 CTC threshold is also used in the NCT00887640 trial that studies the effect of Temsirolimus in CPRC, and a more conventional >5 CTC cut-off point is used in the NCT01682772 trial that explores the use of Olaparib also in the CPRC subpopulation.

Other potential application of CTC in a clinical scenario is as a predictive marker for targeted treatments, in a similar way than KRAS mutations for colon cancer or EGFR mutations in lung cancer. This is the case of the NCT01961843 trial that investigates whether the AR status in CTCs can be used to predict response to Abiraterone. The NCT02012296 attempts the same goal for Enzalutamide and Mifepristone while the NCT01385293 study evaluates the association with the PI3K inhibitor BMK120.

CTC studies have also found a place as surrogate biomarkers in preclinical testing. CTC enumeration has the appeal of providing an early and straightforward test for activity, and also the potential of providing pharmaco-dynamic information (156). A pharmaco-dynamic biomarker provides information "that there is a direct pharmacological effect of a drug", but it does not necessarily provide prognostic or predictive information (157). The value of this test is that it allows the measure of the treatment effect on its targets, if a reliable pharmaco-dynamic test is not affected by treatment, the treatment will be ineffective.

This ability to predict the non-efficacy early in the clinical development of a drug candidate, and the possibility of repeating CTCs at different time points within the development of the disease without the problems associated with repeated biopsies, has the potential of reducing costs, timelines and improve the success rate of the next generation of clinical trials (158,159).

Molecular profiling.

CTC were a strong predictor for overall survival and have predictive value in mCRPC patients. Nevertheless, the majority of the trials have focused on the clinical utility of CTC enumeration, using platforms that detect CTCs expressing epithelial markers. This approach is somewhat simplistic, since excludes tumor stem cells, CTC clusters, and CTCs with mesenchymal or anaplastic phenotypes, which may have important prognostic and predictive implications (160). CTC isolation techniques that select CTCs in a marker-independent manner are under active investigation.

Previous works demonstrated the feasibility of transcriptional and genomic profiling in CellSearch-detected CTC from Prostate cancer patients (111,161) . The molecular and genomic profiling of CTCs may identify novel mutations, shed light on mechanisms of resistance to therapy, and help to predict the likelihood of response to a given therapy, in real time and for a particular patient. In fact, CTCs are entirely different from almost all other biomarkers because they represent a sampling of a patient's tumor, and then can reflect the heterogeneity of metastatic sites.

In this sense, androgen receptor chromosomal amplifications have been detected in CTC from mCRPC patients by FISH. Importantly, the impact of TMPRSS2-ERG rearrangements has also been studied using CTC. FISH detection of ERG rearrangements had a significant association with the magnitude of PSA decline in chemotherapy naïve patients treated with Abiraterone acetate (131). In other study Dittamore *et al*. analyzed 48 samples from 21 mCRPC patients treated with abiraterone plus prednisone (43%) or enzalutamide (57%). No responses were seen in patients with high AR expression on CTC, while 53% of patients with low AR had a PSA decline and stable radiographic disease. Salvi *et al.* studied the role of copy number variations of CYP17A1 and AR genes as predictive biomarkers for outcome of abiraterone treated CRPC patients (162). Those men with both genes amplification had a lower overall survival. In the multivariable analysis both AR and CYP17A1 copy number variations (CNV) independently predicted PFS and AR CNV did it for OS.

AR splice variants lacking the canonical ligand-binding domain are known to be responsible for development of CRPC. Antonarakis *et al.* prospectively determined the presence of AR splice variant 7 messenger RNA (AR-V7) in CTC from patients with CRPC treated with abiraterone (31 pts) or enzalutamide (31 pts). No PSA responses were seen among AR-V7-positive patients either for enzalutamide or abiraterone-treated patients compared to PSA responses in 53% and 68%, respectively, among AR-V7-negative patients (163). PSA, clinical and rPFS and overall survival were also significantly shorter in AR-V7-positive groups.

In a second study the same investigators prospectively enrolled 37 patients with metastatic CRPC initiating docetaxel or cabazitaxel (164). Detection of AR-V7 in CTCs from these men was not associated with primary resistance to chemotherapy. In AR-V7–positive men, taxanes appear to be more efficacious than enzalutamide or

abiraterone therapy, whereas in AR-V7–negative men, chemotherapy and abiraterone or enzalutamide may have comparable efficacy. The authors suggest that circulating tumor cell–based AR-V7 detection could serve as a treatment selection biomarker in CRPC.

These results lead to believe that not only count but also the molecular characterization of CTCs may be of value for response monitoring and drug selection in patients with metastatic prostate cancer.

CTC culture in prostate cancer.

One of the main challenges in the field of CTCs development is the possibility to expand these cells *in vitro*. This would permit a better characterization of CTCs from patients to progress in individualized anti-tumour therapies. Although the CTCs culture *in vitro* remains difficult to achieve, some promising results are emerging and supporting the idea of a new era in the oncology field (165). For example, it has been described that prostate cancer cells are recoverable in murine models using CTC-chip and then able to be cultured *in vitro* (166). Kirby *et al*. captured using GEDI microfluidic CTCs from CRPC and analysed the microtubule response to chemotherapy providing a new strategy for guiding drug selection and the development of individualized treatment (167).

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Chapter 2. Hypothesis

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Patients with metastatic prostate cancer were treated until recent years with hormonal therapy and docetaxel after becoming resistant to hormone deprivation. With the introduction of new agents such as abiraterone, enzalutamide, cabazitaxel or immunotherapy there is an increasing need of novel clinical tools to distinguish patients who will benefit from different therapies. In an ideal environment we would need biomarkers associated with response probability to select which is the most appropriate drug for each patient and in each specific time.

To date, level of prostate-specific antigen have been the most used biomarker to assess the progression in patients with prostate cancer. However, in many cases, blood levels of PSA does not accurately reflect the state of progression of the disease or the risk for new metastasis development. In addition, radiological evaluation is difficult and largely unhelpful in patients with mCRPC. Therefore, effective treatment of metastatic disease requires clinical tools to select and monitor therapy.

Taking into account this scenario, there are two priorities to improve the clinical management of metastatic CPRC patients: the characterization of prognostic, follow-up and therapy response predictors to guide the clinical intervention and the identification of new therapeutic targets to increase the treatment options of these man.

Due to the continuous evolution of tumors, which involves genetic and epigenetic alteration of cancer cells and tumor heterogeneity, it is well accepted that primary tumors and individual metastases provide a limited information of the molecular status of cancers. In this sense, **CTCs** provide a real-time and sequential **''liquid biopsy''** for metastasic cancer patients. These cells can provide significant information for a better understanding of tumor biology and tumor cell dissemination.

Into this context of knowledge, our hypothesis is that, in addition to the clinical value of CTCs counting to predict mCRPC patient outcome, the molecular characterization of the CTC population from these patients offers a unique source to obtain important information on:

1) **Patients prognostic.**

2) **Patients monitoring** to be able to determine early response, anticipating and improving the biochemical and radiological evaluation.

3) **Therapy selection**, identifying the mechanisms of resistance to current therapies.

4) **Therapy development**, providing information of the main actors for mCRPC progression and aggressiveness.

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Chapter 3. Objectives

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The objectives of this thesis are set to try to explore the possibilities for the use of CTCs in the management of patients with cancer metastatic castration-resistant prostate. We have established three main objectives:

1. The assessment and quantification of CTCs in a cohort of patients with metastatic CRPC and treated with docetaxel/cabazitaxel in order to evaluate the role of CTC count as an independent prognostic and monitoring marker.

2. The application of a new methodology for CTCs analyses in CRPC, with high sensibility and versatility to provide a robust alternative to the CellSearch, the actual reference system.

3. The molecular characterization of CTCs present in patients with mCPRC using a global gene expression approach in order to identify new specific CTCs markers and potential genes related to resistance to chemotherapy.

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Chapter 4. Material and methods

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1. Study design.

We conducted a prospective, longitudinal study in order to analyse the number and the biology of CTCs from peripheral blood of mCRPC patients treated at first line chemotherapy with either intravenous docetaxel 75 mg/m^2 or cabazitaxel 25 mg/m^2 every 3 weeks. The study was approved by the research ethics committee and was performed in accordance with the ethical standards of the Declaration of Helsinki. Informed consent was signed by all patients previously the inclusion in the study. The informed consent model and the ethical research committee approval are shown in Annex 2 y 3.

CTCs counts were assessed using CellSearch system at baseline, before the 3rd cycle and the 6th cycle during the chemotherapy treatment. Two additional samples for CTCs molecular characterization were taken before starting chemotherapy and when PSA or radiological progression was established (Figure 4.1). Treatment was continued until progression, unacceptable toxicity, death or when the number o preplanned cycles of chemotherapy was completed (minimun of 6 or maximum of 9 cycles).

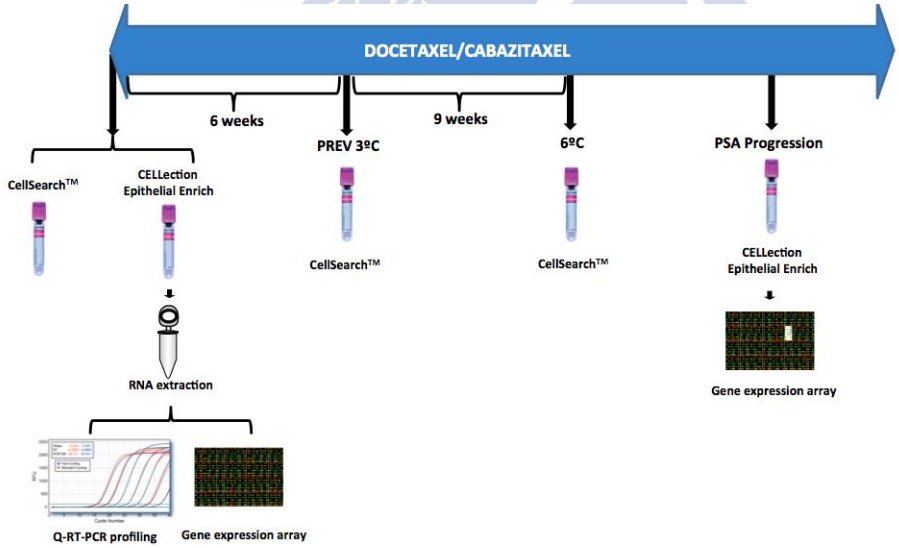


Figure 4.1. Study design.

2. Patient inclusion.

A total of 29 mCRPC patients and 15 healthy individuals were prospectively enrolled in this study from January 2012 to April 2014 at the Medical Oncology Department in the Hospital Universitario de Santiago de Compostela, Spain. The inclusion and exclusion criteria are listed in Table 4.1. Control group included 15 healthy men with similar ages range to patients and no previous cancer episodes.

A radiologist independently reviewed the imaging datasets, blinded to CTC results. According to RECIST 1.1 criteria, patients with non-measurable disease only at baseline, e.g., bone lesions were allowed. Response was determined by biochemical (PSA values), radiological (CT scan, bone scan) and clinical criteria, evaluated by a genitourinary oncology physician.

Table 4.1. Study inclusion and exclusion criteria.

Inclusion criteria
Patients with metastatic prostate adenocarcinoma
Progression to hormonal therapy. Indication of chemotherapy
Patients with serum castration levels of testosterone (testosterone <50 ng/dL or <1.7 nmol/L), PSA progression and/or clinical progression to castration, or progression despite anti-androgen withdrawal for at least 4-6 weeks
No measurable disease is required
ECOG PS 0-2
Ability to understand the procedures of the study and informed consent
Estimated overall survival > 3 months
Exclusion criteria
Non-adenocarcinoma prostate cancer
PSA progression but no evidence of metastases
CNS metastases
ECOG PS> 2
Inability to understand the study procedures and to signed an informed consent
Estimated overall survival <3 months

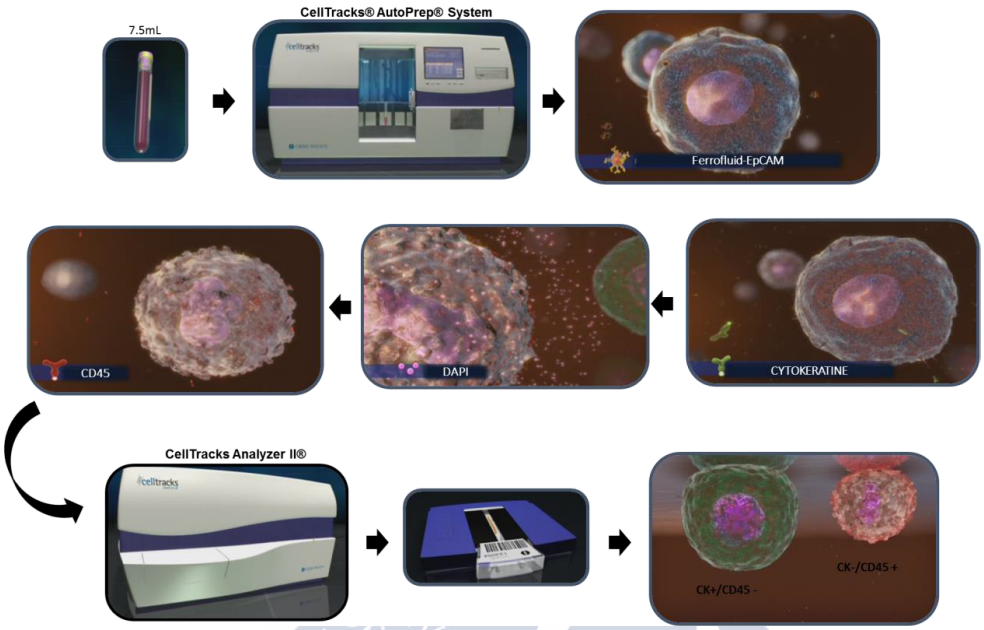
3. Blood sampling and CTC analyses.

3.1 CTC isolation and quantification using CellSearch system.

CTC analyses were performed using CellSearch (Veridex LLC, Raritan, NJ, USA) technology. Samples were drawn into 10 mL evacuated blood drawtubes (CellSave Preservative Tubes, Veridex LLC, Raritan, NJ, USA), maintained at room temperature and processed within 96 h after collection. 7.5 mL of peripheral blood, together with 6.5 mL of sample buffer (Veridex LLC, Raritan, NJ, USA), were centrifuged at 800xg, 10 min at room temperature. After centrifugation, cells expressing EpCAM were

immunomagnetically enriched and fluorescently labelled with DAPI, CD45-APC, and CK-PE by CellTrack AutoPrep system (Veridex LLC, Raritan, NJ, USA)(Figure 4.2).

A



B

C

Figure 4.2. CTC analysis with the CellSearch system. A. Representative scheme of CTC isolation and characterization process that CellSearch system applies to blood samples from patients. B. CTC image obtained after the analysis of a blood sample from a mCRPC patient with the CellSearch system. Cells were considered CTC when they have round-oval morphology, nucleated (DAPI+, ≥ 4µm), lacking CD45 and expressing CK-PE (CK8, 18 and 19). C. Leucocyte, showing DAPI and CD-45 expression (CK-PE negative).

Then the images of stained cells were acquired by a semiautomatic fluorescence microscopy system, CellTrack system (Veridex LLC, Raritan, NJ, USA). Finally, two experimented reviewers selected the morphological intact CTC, defined as round-oval morphology, nucleated (DAPI+), lacking CD45 and expressing CK, from the gallery of objects proposed by the system.

Concomitantly, PSA, lactate dehydrogenase and alkaline phosphatase were analyzed as routine markers at the clinical site.

3.2 CTC isolation using CELLection and molecular characterization by RT-qPCR.

Gene expression analysis was carried out on blood samples extracted prior to initiating chemotherapy (Figure 4.3). The protocol combines an EpCAM-based CTC inmunoisolation and a RT-qPCR analysis of a previously pre-amplificated genetic material (168). CTC isolation was made with CELLection^{TM} Epithelial Enrich system (Invitrogen, Dynal, Oslo, Norway) that contains beads coated with EpCAM antibodies. 15 ml of Buffer 2 were added to 7.5 ml of peripheral blood (PBS 1x, BSA 0,1%, EDTA 2 mM). After sample centrifugation at 1250xg during 15 min at room temperature plasma fraction was discarded. Then, 7.5 ml of Buffer 2 were added to 100 µl of EpCAM-conjugated beads. They were incubated at 4ºC, 30 min at constant rotation. After that, cells linked to the magnetic beads were recovered using a magnet. The recovered fraction was then washed three times with Buffer 1 (PBS 1x, BSA 0,1%, pH7.4) to decrease the unspecific isolation. Finally, isolated cells were re-suspended in 100 µl of RNA later (Ambion®, Austin, USA) to avoid RNA damage and were stored at -80ºC until use.

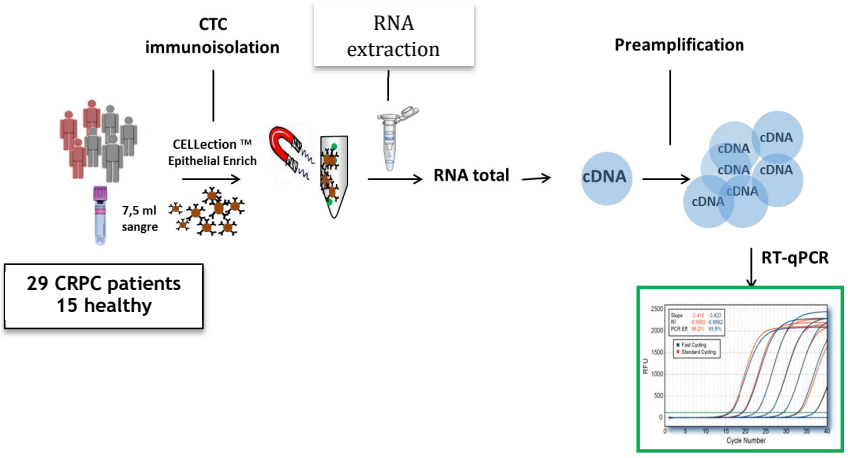


Figure 4.3. Representative scheme of CTC isolation and the different steps required to perform the gene expression analysis by RT-qPCR.

Total RNA from CTC was extracted with the QIAmp viral RNA mini kit (Qiagen, Valencia, CA, USA), designed for very low cellularity samples following the manufacturer´s instructions. cDNA was synthesized by using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and subjected to a preamplification for 14 cycles with TaqMan® PreAmp Master Mix kit (Applied Biosystem, Foster City, CA, USA) prior to RT-qPCR (TaqMan Gene Expression Assays; Applied Biosystems), to maximize detection rates.

We analyzed in the CTCs fraction the expression of 15 candidates genes selected in basis on their relevance for prostate cancer biology plus the house-keeping gene *GAPDH* as total cellular load marker and *CD45*, a specific gene for hematopoietic cells, to estimate non-specific isolation. The genes selected for this study have been previously related to androgen-regulation (*AR, CYP19A1* and *CYP17A1*), stem cell phenotype (*CD133, CD44, ALDH1A, ABCG2* and *CD49f*) and prostate cancer aggressiveness and/or resistance to taxanes (*BIRC5, CLU, GDF15, RAB7A, SPINK1, TUB1A1, MDR1*) (Figure 4.4).

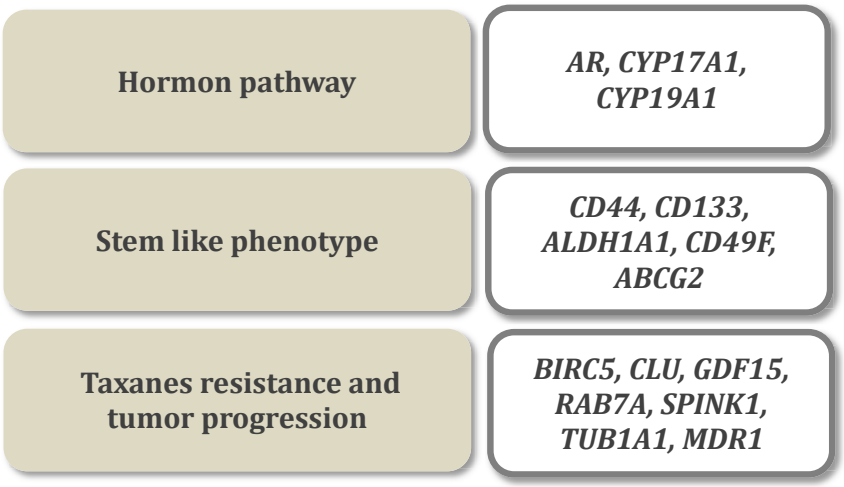


Figure 4.4. General fuctions of candidate genes analyzed by RT-qPCR.

RT-qPCR was performed using TaqMan® Gene Expression Assays and StepOnePlus Real-Time PCR System (Applied Biosystem, Foster City, CA, USA). cDNA was diluted at 1:20 with TE1X. Then, 5 μl of this dilution were added to 10 μl TaqMan® Master Mix, 1 µl TaqMan® Gene Expression Assay, 4 µl RNase free water. PCR conditions were: 50ºC, 2min; 95ºC, 10 min; (95ºC, 15 sec; 60ºC, 1min) x 40 cycles.

Data were analyzed using StepOne Software v.2.1. (Applied Biosystems) and expression values were normalized to CD45. The same protocol was applied to healthy volunteer's blood.

3.3 Whole gene expression analysis of CTCs.

Whole gene expression analysis was carried out on blood samples extracted from 9 patients prior to initiating chemotherapy and when progression was confirmed. In parallel, the same protocol was applied to blood samples from 6 healthy donors stablish the baseline of background from unspecific immunoisolation (Figure 4.5). CTCs isolation and RNA extraction were made as described above in section 3.2 of Material and Methods.

Gene expression arrays. Total RNA extraction, complete Whole Transcriptome Amplification (WTA2, Sigma Aldrich, Sant Louis, USA) and gene expression array was performed as described (168). Briefly, total RNA was extracted with the QIAmp viral

RNA mini kit (Qiagen, Valencia, CA, USA) specifically designed for very low cellularity samples.

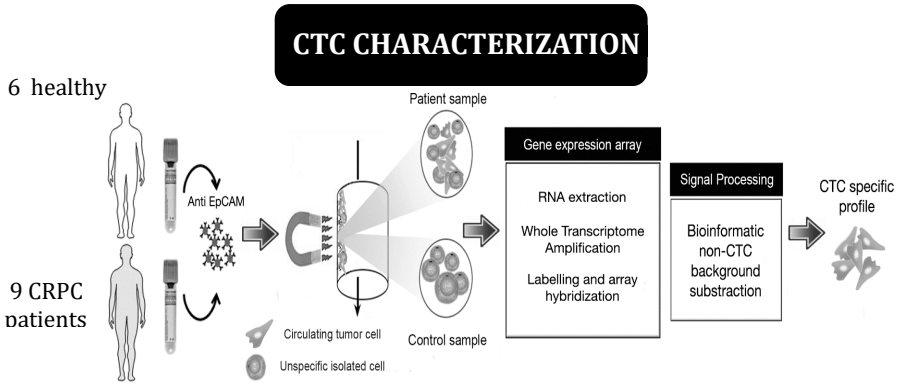


Figure 4.5. Schematic representation of the procedure used for CTCs gene expression profiling. Taken from Mariscal *et al.* (under review).

Subsequent pure RNA was then subjected to Complete Whole Transcriptome Amplification PCR for 20 cycles using the maximum amount of RNA; Cy3 labeling and hybridization onto Agilent 4x44k gene expression arrays. Upon hybridization, signal was captured and processed using an Agilent scanner (G2565B, Agilent Technologies). The scanner images were segmented by the Agilent Feature Extraction Software (v9.5) with the protocol GE1-v5_95.

4. Statistical analysis.

Data were analysed using SPSS (Chicago, version 15.00 for Windows) and GraphPad Prism 4.00 software (GraphPad Softwares Inc, San Diego, CA, USA). p values <0.05 were considered statistically significant. For the collection and analysis of clinical data in the patient group a database was designed. The main variables collected are summarized in annex 1.

Regarding the experimental variables, CTC counts obtained by CellSearch are presented as number per 7.5 ml of blood while the expression of the candidate markers analysed in CTCs by RT-qPCR are presented as 40-Ct normalized by CD45 expression (40-Ct). The cut-off value to categorize the expression levels of the markers was calculated on basis of percentile that best grouped patients into good and poor evolution groups.

The association between categorical variables were done using 4x4 tables and the Chi-square test for significance or the Fisher exact test if the expected values in each group were <5. T-test for independent samples was used to compare the mean of the different clinical and experimental groups. For multivariate analyses, biochemical response was defined as PSA decrease of ≥50 %. Bivariate correlation analysis was carried out according Pearson statistic.

Regarding survival analyses, PFS and OS were analyzed using Kaplan-Meier analysis and differences were examined by log-rank test. Univariate and multivariate analyses were performed using Cox regression statistics.

Gene expression arrays analysis. Extended dynamic range implemented in the Agilent software was applied to avoid saturation in the highest intensity range. The Agilent feature extraction was used as raw data for further pre-processing. The processed signal (gProcessed-Signal) value was chosen for the statistical analysis instead of the signal with substracted background (gBGSubSignal) since it produces lower average coefficient of variation (CV) in Spike-In and gene replicates (169,170). Spatial Detrend correction was applied using the Agilent Feature Extraction algorithm. The following features and/or genes which did not conform to the established quality criteria were filtered: (a) non-uniform pixel distributed outliers and population replicate outliers according to the default Agilent feature extraction criteria; (b) spots not differentiated from background signal; (c) spots in the range of negative controls.

After this filtration, we considered CTC-associated genes those non detected in any control and detected in at least 5 patients at baseline and progression. In order to compare the profile expression in CTC fraction at baseline and progression normalization between microarrays was carried out using the Quantile method implemented in the Limma package of the R statistical software version 3.2. After normalization, the two quantiles 0.25 (Q1=361.78) and 0.75 (Q3=2953.91) of all gene signals in all experimental conditions were computed. The resulting list contains all genes which expression was lower than Q1 at baseline and higher than Q3 at progression.

Finally, gene set characterising CTC population was analysed with Ingenuity Pathway Analysis software (IPA; Qiagen, Redwood City, CA, USA) for networks generation and the identification of the main signalling pathways involved in CTCs biology.

Chapter 5. Results

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5.1. Patient characteristics and treatment efficacy.

Between January 2012 and April 2014 29 mCRPC patients were prospectively enrolled at the Medical Oncology Department in the Hospital Universitario de Santiago de Compostela. Samples were acquired from 15 healthy controls using the same institutionally approved protocol. Patient baseline demographics and clinical characteristics are described in table 5.1 and table 5.2. Twenty seven percent of patients debuted as stage IV disease. Those with local or locoregional prostate cancer were treated by prostatectomy (20%) or radiotherapy (41%). Most of patients received 2 or 3 hormonal maneuvers, including at least complete blockade and antiandrogen withdrawal. Mean serum PSA at initial diagnosis was 258 ng/mL (range 2-2327).

Table 5.1 Patient demographics and clinical characteristics I.

Comorbidities, n (%)	
COPD	4 (13.8)
Hypertension	9 (31)
Diabetes mellitus	3 (10.3)
Hypertension + Diabetes	3 (10.3)
None	9 (31)
Initial diagnosis	
Stage, n (%)	
I	1 (3.4)
IIA	6 (20.6)
IIB	4 (13.8)
III	8 (27.5)
IV	8 (27.5)
PSA, ng/mL	
Mean, range	258 (2-2327)
Median, range	11 (2-2327)
Initial treatment, n (%)	
Radical prostatectomy	6 (20.6)
Radiotherapy	
Radical	12 (41.2)
Adjuvant	2 (6.5)
Palliative	3 (10.3)
Previous Hormonal Maneouvers, n (%)	
1	1 (3.4)
2	14 (48.2)
3	10 (34.4)
4	4 (13)

COPD: Cronic Obstructive Pulmonary Disease.

Gleason score was >7 in 37.9% of cases. All patients had bone metastases; approximately one-third of the study population had lymph nodes metastases, and 13% in the lung. Twenty-six men received docetaxel and 3 cabazitaxel. Since our center participated in the phase III FIRSTANA trial, comparing the efficacy of cabazitaxel versus docetaxel in first line mCRPC, we decided to allow the inclusion in our study of 3 patients randomized to the cabazitaxel arm.

Table 5.2 Patient demographics and clinical characteristics II.

Age, years	
Median (range)	69.6 (52-80)
ECOG PS, n (%)	
0	7 (24.1)
1	19 (65.5)
2	3 (10.3)
Weight, Kg	
Mean	83.4
Size, cm	
Mean	163
Gleason score, n (%)	
≤7	15 (51.7)
>7	11 (37.9)
Unknown	3 (10.4)
Disease site, n (%)	
Bone	29 (100)
Bone only	15 (51.7)
Lymph node + Bone	10 (34.4)
Lymph node + Bone + Lung	2 (6.5)
Lung + Bone	2 (6.5)
Biphosphonates, n (%)	27 (93.1)
Radionuclides, n (%)	1 (3.4)
PSA, ng/mL	
Mean, range	417 (12-3238)
Median, range	121 (12-3238)
AP, IU/L	
Mean, range	566 (77-3115)
Median, range	320 (77-3115)
LDH, IU/L	
Mean, range	511 (121-1136)
Median, range	454 (121-1136)
Chemotherapy	
Docetaxel, n (%)	26 (89.7)
Cabazitaxel, n (%)	3 (10.3)
Number of cycles, median	6
Dose intensity, %	95
CPRC Second line treatment, n (%)	
Abiraterone	14 (48.2)
Cabazitaxel	5 (17.2)
Docetaxel	2 (6.9)
Vinorelbine	1 (3.4)
Ciclophosphamide	1 (3.4)
Best supportive care	4 (13.8)
None	2 (6.9)

PSA: Prostate specific antigen; AP: Alkaline phosphatase;
LDH: lactate dehydrogenase.

Treatment summary and efficacy.

Median number of chemotherapy cycles was 6. Most patients had no dose reductions while 3 participants in the study had chemotherapy dose delays. About 80% of patients received additional post-study therapy. The most frequent types of post-study therapy received were abiraterone (48.2%) and chemotherapy (30.9%).

The median PFS was 7.4 months (95% CI, 5.9–8.7) (Figure 5.1). Median OS was 27.3 months (95% CI, 16–38.7) (Figure 5.2). A PSA decline of ⩾50% from baseline occurred in 55.2% of patients (Table 5.3). In the subset of men with measurable disease the disease control rate (defined as CR + PR + SD) for all patients was 48%.

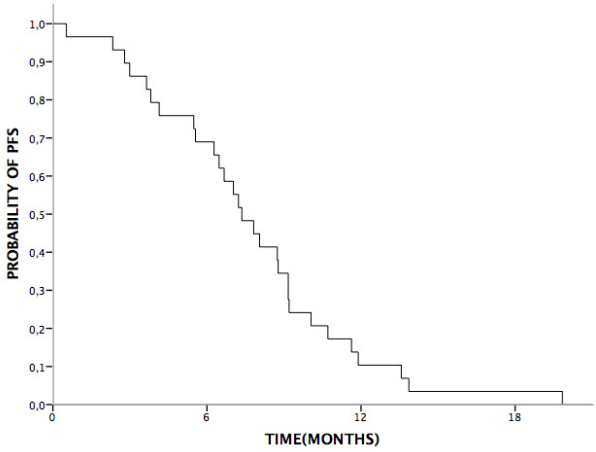

Figure 5.1. Kaplan-Meier plot for PFS of all the patients included in the study.

Table 5.3. Efficacy results.

Progression free survival	
Median, months	7.4
95% CI	5.9-8.7
Overall survival	
Median, months	27.3
95% CI	16.0-38.7
PSA response	
Partial response, %	55.2
No changes, %	13.8
Progressive disease, %	27.6
No evaluable	3.4
Radiological response	
Partial response, %	0
No changes, %	48
Progressive disease, %	24
No evaluable	27

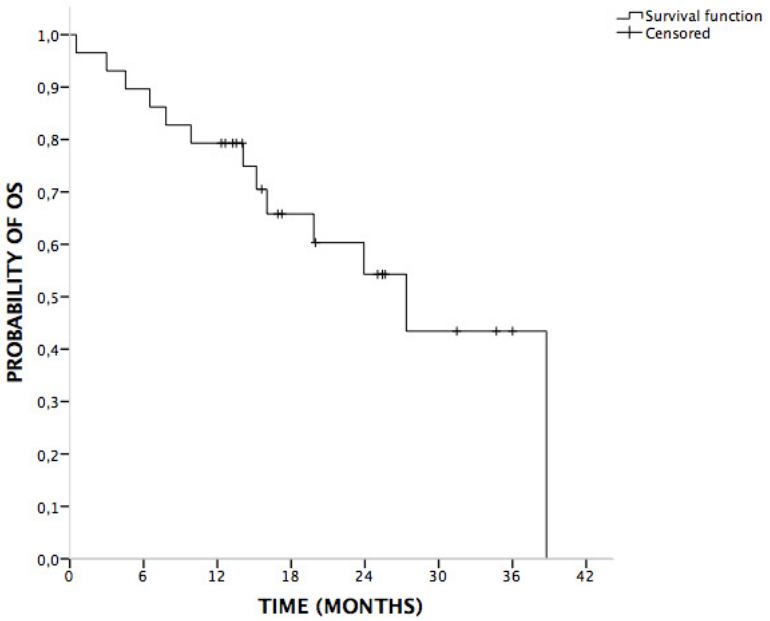

Figure 5.2. Kaplan-Meier plot for OS of all the patients included in the study.

Safety.

Regardless of causality, fatigue (any grade) was the most frequent adverse event (Table 5.4). Among the non-haematologic adverse events (AE), the only grade 3-4 toxicity corresponded to dacryocystitis in one patient. The incidence of diarrhoea, neuropathy and nausea occurred in 34.4 %, 31% and 24.1% of patients, respectively (Table 5.4).

Table 5.4. Chemotherapy toxicity.

	Any G , (%)	G3, (%)	G4, (%)
Non-haematologic AE			
Fatigue	26 (89.7)	0	0
Alopecia	20 (69)	0	0
Dacryocystitis	1 (3.4)	1 (3.4)	0
Onycholysis	8 (27.5)	0	0
Cutaneous	1 (3.4)	0	0
Peripheral edema	3 (10.3)	0	0
Anorexia	4 (13.8)	0	0
Diarrhoea	10 (34.4)	0	0
Nausea	7 (24.1)	0	0
Vomiting	3 (10.3)	0	0
Neuropathy	9 (31)	0	0
Haematologic AE			
Anaemia	24 (82.8)	2 (6.9)	0
Neutropenia	7 (24.1)	1 (3.4)	3 (10.3)
Thrombocytopenia	2 (6.9)	2 (6.9)	0

Regarding hematological side effects anemia was the most common, but mostly grade 1-2. Seven and four patients had grade 1-2 and grade 3-4 neutropenia, respectively.

5.2 Clinical value of CTC counts monitoring for mCRPC management.

CTC counts were analyzed in a total of 78 samples. CTCs were detected in 93.1 %, 55.1% and 31% of patients at baseline, at 3 rd and at 6th cycle, respectively. At the time of this analysis, all patients had progressed and 44.8 % (n = 13) of them had died. Concomitant serum PSA and radiological assessments were evaluable in 28 and 21 patients, respectively. Table 5.5 summarize the number of patients in whom CTC were analyzed at baseline, at 3rd and at 6th chemotherapy cycle as well as the mean, median and range of these counts.

Table 5.5. Description of CTC values during treatment.

Characteristic	Baseline CTC (CTC0)	CTC cycle 3 (CTC3)	CTC cycle 6 (CTC6)
N Valid Missing	29 0	28 1	21 8
Mean	159.38	57.3	9.5
Median	12	4	0
Typical deviation	561	261.8	19.9
Range	0-3000	0-1392	0-66

Many authors have previously described that patients with high CTC counts have higher frequency of visceral metastases, high Gleason score and other unfavorable clinico-pathological features. In these studies, a cutoff of 5 CTC was chosen to be correlated with variables such as age, Gleason score, site of metastasis, etc ... (Table 5.6). Despite the limitations associated with the small size of our group of patients, we conducted a description of the clinicopathological features in patients with < 5 or ≥ 5 CTCs per 7.5 ml of blood (Table 5.6 and Figure 5.3).

We found higher number of CTCs in patients diagnosed with locally advanced disease (28 vs 263; p=0.054), nodal invasion (33 vs 443; p=0.03), and also in patients that were responsive to hormonal therapy for less than 24 months (18 vs 311; p=0.04). Mean levels of serum alkaline phosphatase and PSA were significantly higher in the CTC high subpopulation.

Table 5. 6 Clinical characteristics of the CTC high and CTC low subpopulations (CTC0).

Characteristic	< 5 CTC	≥5 CTC	p
N	10	19	
Age	71.4	68.74	0.433
Age at diagnosis	66.0	63.9	0.325
Locally advanced disease at diagnosis	44%	70%	0.212
Nodal invasion (cN)	22%	41%	0.012
Gleason score >7	33%	47%	0.40
Hormonal therapy, years	3.41	2.26	0.221
Visceral disease	11%	23%	0.187
Baseline PA, mean	207	765	0.03
Baseline LDH, mean	396	558	0.216
Baseline PSA, mean	107	580	0.03

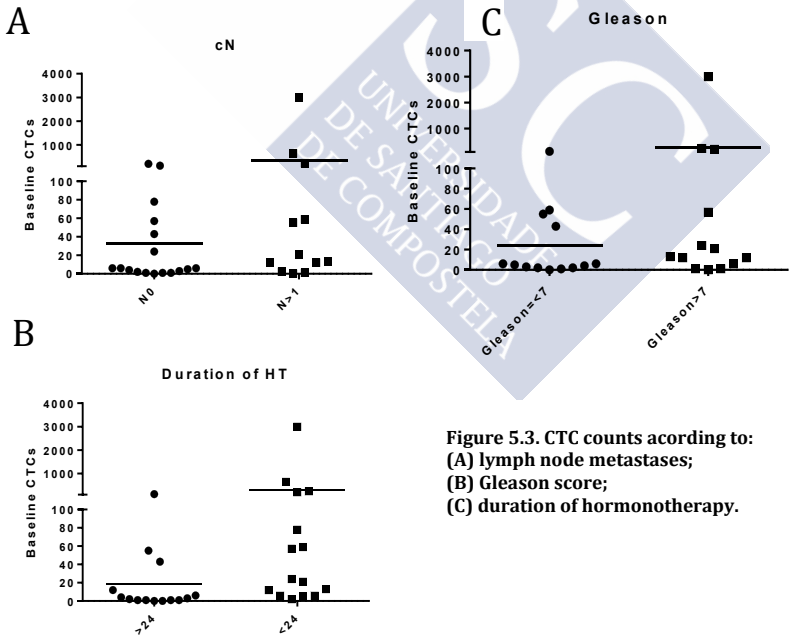

Figure 5.3. CTC counts acording to:
(A) lymph node metastases;
(B) Gleason score;
(C) duration of hormonotherapy.

Association of CTC counts with OS.

The prognostic value of categorical CTC counts (<5 vs. ≥5 CTCs) to predict OS was analyzed in a landmark analysis at each time point of CTC assessment. Kaplan–Meier analyses revealed significant differences in median OS times for all time points. Median survival times were 16 months (95 % CI 9.4-24.7) for those patients with ≥5 CTCs at baseline versus not reached for those < 5 CTCs (Figure 5.4 and Figure 5.5).

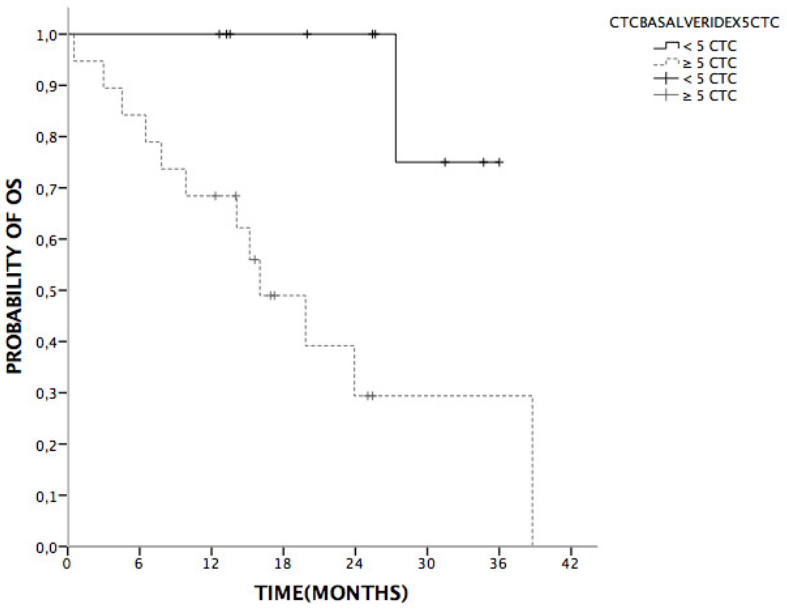

Figure 5.4. Kaplan-Meier analyses for overall survival according to categorical CTC counts (log-rank =0.007).

In the analysis of Receiver Operating Characteristic (ROC) curves, baseline CTCs count was the risk factor with higher AUC for predicting the possibility of having an above mean overall survival (AUC=0.784). At the same time higher CTC counts were inversely proportional to the probability of biochemical response (AUC=0.321). There was only a weak association between CTCs and PFS. Phosphatase alkaline (AUC=0.727) and LDH (AUC=0.636) levels had better correlation with survival than PSA (AUC=0.530).

We also evaluated the number of CTCs present before and after chemotherapy (before 3rd cycle and before the 6th cycle) in patients that showed early progressive disease, to

see if the early increase in CTCs can anticipate tumor progression. We found that patients with CTCs ≥ 5 at 3rd cycle had a very high risk of being in biochemical (HR=5.571; p=0.055) or radiological progression (HR=10.679; p=0.01). Patients with ≥ 5 CTCs at the 6th cycle had 28 (p=0.04) and 12.35 (p<0.01) more risk of biochemical and radiological progression, respectively (Table 5.7).

To find out whether this fact was related to tumor progression, we analyzed the CTCs levels during chemotherapy. All patients who progressed during treatment had ≥ 5 CTC at baseline determination, allowing discard this assumption. By contrast patients who started from a baseline count ≥ 5 CTC, but after chemotherapy became < 5CTC achieved a PFS (8 months) and OS (38 months) similar to the group that started with < 5 CTC at baseline determination.

Table 5.7 Association between CTC values and biochemical and radiological progression.

≥ 5 CTCs	OR Bq Progression	p	OR Rx progression	p
Baseline	5.2	0.196	1.66	0.367
After 3 cycles	5.57	0.055	10.769	0.01*
After 6 cycles	28	0.04*	12.35	<0.01*

Bq: biochemical; Rx:radiological. *p< 0.05.

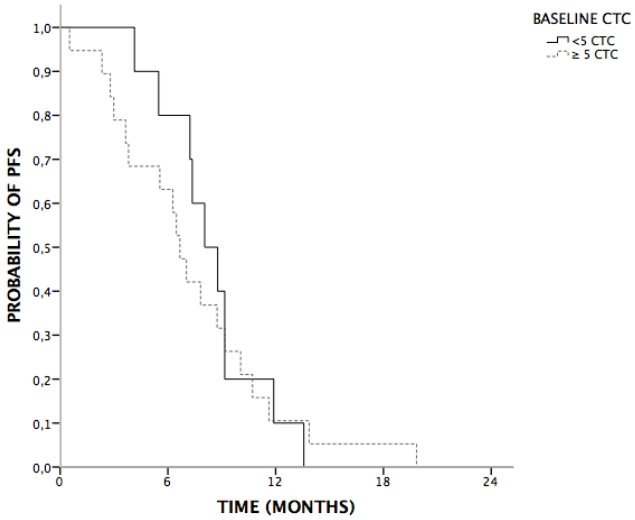

Figure 5.5. Kaplan-Meier analyses for PFS according to the CTC status at baseline (log-rank=0.73)

CTC count and chemotherapy.

Mean number of CTCs decreased after treatment from 159.38 to 57.3 before the 3rd cycle and to 9 before the 6th cycle. The overall rate of biochemical response was 55% (47% in the ≥5CTC baseline subgroup vs 70% in the <5CTC baseline CTC subgroup), but those patients that converted to <5CTC after chemotherapy had similar response rates to those with <5CTC at baseline (73% vs 38%) (Figure 5.6 and Figure 5.7).

Patients with ≥5CTC at baseline had a higher risk of progression at 12 weeks (36%, compared to 10% for those men with <5CTCs) and a lower mean PFS and OS. On the other hand patients with early conversion from ≥5CTC to <5CTCs after 3 cycles of docetaxel had similar PFS and OS to those patients with low baseline CTC counts. Patients with higher baseline CTC counts could represent a heterogeneous subgroup with a subset of chemosensitive patients that derive the most benefit from docetaxel treatment.

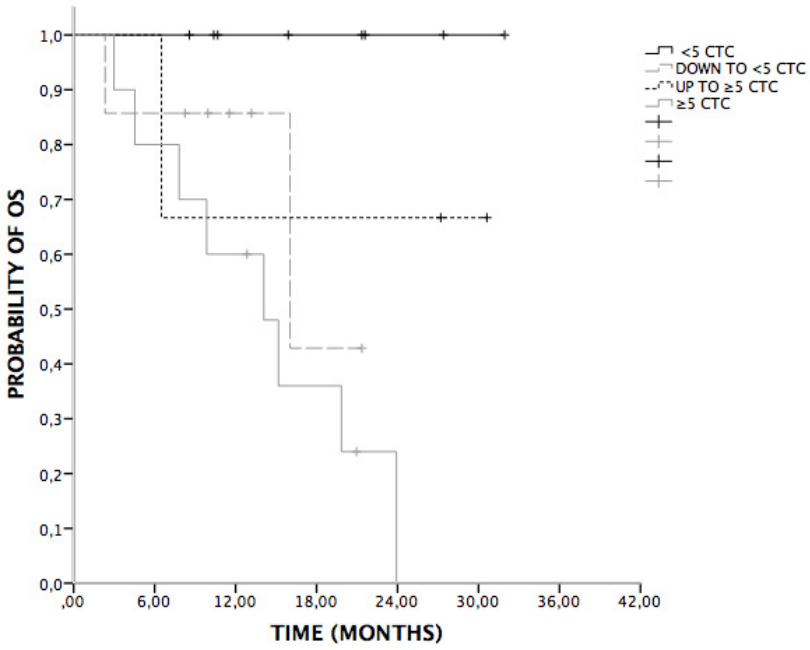

Figure 5.6. Kaplan-Meier analyses for overall survival according to categorical CTC counts (<5CTCs baseline; from ≥5CTCs to < 5CTC after chemotherapy; from <5 CTCs to ≥ 5 CTCS after chemotherapy; ≥ 5 CTCs baseline).

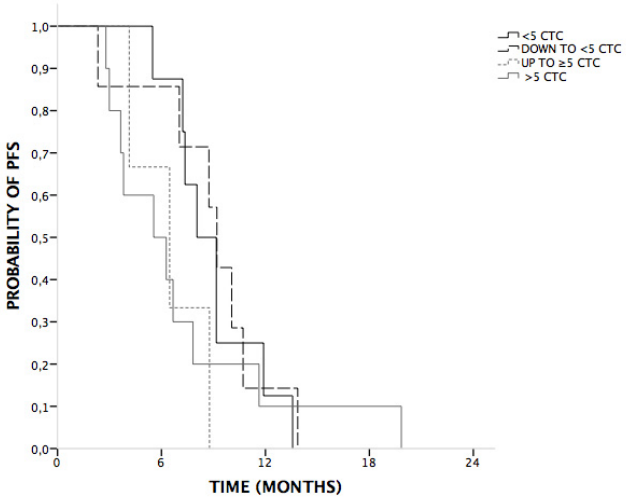

Figure 5.7. Kaplan-Meier analyses for progression free survival according to categorical CTC counts (<5CTC baseline; from ≥5CTC to <5CTC after chemotherapy; from <5 CTCs to ≥5 CTCS after chemotherapy; ≥5 CTCs).

5.3. A new approach for CTCs analysis in mCRPC patients based on immunoisolation and RT-qPCR.

CTCs immunoisolation from peripheral blood in mCRPC patients.

Immunoisolation of CTC from peripheral blood samples was performed with magnetic beads coated with EpCAM antibodies, as a well accepted strategy for prostate carcinomas (107,171). After CTCs immunoisolation we analysed the enriched fraction by q-RT-PCR. For that, after RNA extraction we performed a pre-amplification step to increase the detection rate of the PCR. First, we evaluated the expression levels of *GAPDH* as a marker of cellularity, which includes both CTC and unspecific blood cells, normalized to the background of *CD45*expression as specific marker for cells of hematopoietic origin (168). As shown, *GAPDH* levels were significantly higher in the group of patients compared to controls (Figure 5.8 A) indicating the presence of an extra population of cells isolated from the blood of CRPC patients.

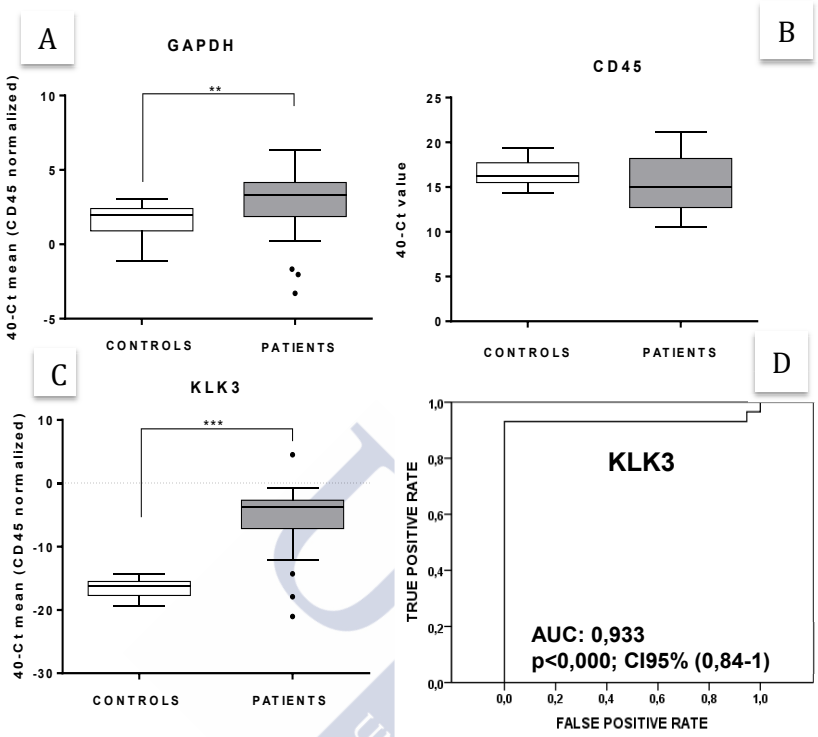

Figure 5.8. Validation of the CTC isolation approach in mCRPC patients. Box plots indicate median values in the control group compared with the group of mCRPC patients for GAPDH (A) CD45 (B) and KLK3 (C) normalized to CD45. CD45, used as a marker of unspecific blood cells isolation showed no differences between both groups, while GAPDH and KLK3 demonstrated optimal accuracy for CTC detection (**p< 0.01; *** p< 0.01). (D) ROC-curve showing the high sensitivity and specificity of *KLK3* to detect the presence of CTCs in our mCRPC cohort. CD-45: cluster of differentiation 45; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; KLK3: kallikrein 3; AUC: area under curve.

In addition, *CD45* did not present differences between both groups (Figure 5.8 B), demonstrating that the unspecific background resulting from the process of immunoisolation was similar in the group of patients and controls. Importantly, when we compared the expression of *KLK3*, as a specific marker for prostate cells, no positive cases were found in the group of controls while 93.1 % of patients were positive for *KLK3*, reinforcing the high specificity of our strategy for CTCs detection and analysis (Figure 5.8 C and D). Globally, these results demonstrated the presence of CTC in our cohort of CRPC patients.

CTCs profile in CRPC patients.

Once demonstrated the efficiency of the CTCs isolation strategy, we explored the gene-expression profile of CTCs in samples from CRPC patients. For this, we analyzed relevant genes for prostate cancer progression related to androgen-regulation (*AR, CYP19 and CYP17*), stem cell phenotype (*CD133, CD44, ALDH1A, ABCG2 and CD49f*) and prostate cancer aggressiveness and/or resistance to taxanes (*BIRC5, CLU, GDF15, RAB7A, SPINK1, TUB1A, MDR1*). We analyzed the expression levels of these genes in the whole set of patients and controls, and identified those genes with a significant expression in CTC from the group of patients compared to the background of unspecific isolation from the controls (Figure 5.9).

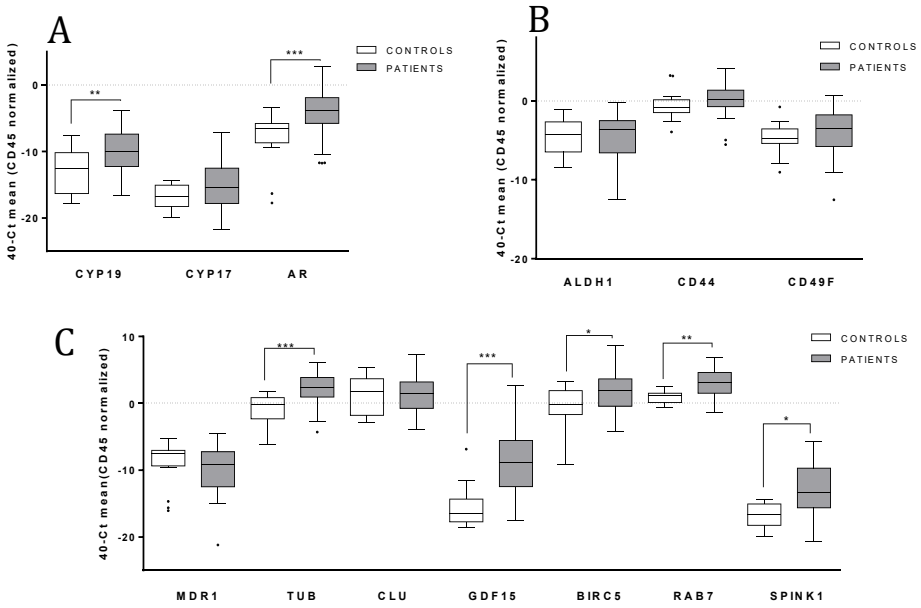

Figure 5.9. Gene expression profiling in CTCs from mCRPC patients. Significant expression levels of genes involved in relevant signalling pathways for PCa biology: **(A)** hormone pathways **(B)** stem cell features and (C) associated with prostate cancer progression and chemotherapy resistance. White boxes represent the gene expression levels in the group of healthy controls, grey boxes correspond to patients (Mann Whitney test, *p<0.05; **p<0.01***p<0.001).

CD133 and *ABCG2* were expressed in less than 30% of patients and 20% of controls, therefore, they were discarded for further analyses. Among the remaining genes, we found significant higher expression levels in patients for *AR*, *CYP19*, *BIRC5*, *TUB1A*,

GDF15, *RAB7* and *SPINK1.* All of them are considered to characterize the population of CTC in our cohort of patients. This concern was reinforced after the analysis of ROC curves, showing all the validated genes high areas under the curves or AUROC ranged from 0.70 (*BIRC5*) to 0.87 (*GDF15*) (Table 5.8).

TABLE 5.8. Diagnostic value to detect disseminated disease in mCRPC patients.

Receiver Operating Characteristic (ROC) curves			
GENES	**AUC**	**p-value**	**CI 95%**
AR	0.76	0.002	0.62-0.90
CYP19	0.74	0.006	0.59-0.80
TUB1	0.83	<0.001	0.72-0.95
GDF15	0.87	<0.001	0.76-0.98
BIRC5	0.70	0.024	0.54-0.86
RAB7	0.81	0.001	0.68-0.94
SPINK1	0.79	0.001	0.66-0.92

Taking into account these results, the CTC population of our mCRPC cohort is better characterized by genes implicated in hormone synthesis and signalling together with genes strongly associated with proliferation, cell adhesion, immune system evasion and also the response to treatment based on taxanes. Importantly, the global CTC population of mCRPC was not found positive for the stem like markers analyzed**.**

Association between the CTCs profile and clinical parameters.

We analysed the possible association between standard clinical parameters and the levels of our CTC-markers and we found the results summarized in Table 5.9. Patients with PS = 0 had significant lower levels of *AR, SPINK1* and *GDF15*. Patients with Gleason score ≥7 were characterized by higher levels of *TUB1* while the presence of lymph node metastasis was not correlated with higher levels of these CTC-markers.

Patients who received fewer treatments before docetaxel or cabazitaxel administration had higher levels of some markers, reaching statistical significance for CYP19 and *GDF15*. Interestingly, serum PSA levels at baseline correlated with PSA (*KLK3*) levels analysed in CTC population. On the other hand, patients with high levels of LDH and PA at baseline were those with high levels of *GDF15* marker. Overall, these results seem to reflect the presence of greater number of CTCs in patients with poor clinical status before the treatment onset, in terms of PS, Gleason score and biochemical markers.

Table 5.9. Correlation between baseline characteristics and CTCs profile

	KLK3 (mean)	AR (mean)	TUB1 (mean)	CYP19 (mean)	BIRC5 (mean)	RAB7 (mean)	SPINK1 (mean)	GDF15 (mean)
ECOG								
0 (n=7)	-7.69	-7.16*	3.28	-11.44	1.53	3.59	-16.55*	-13.43*
1-2 (n=22)	-5.17	-3.49*	1.75	-9.42	1.98	2.93	-11.92*	-6.85*
Gleason score								
≤7 (n=15)	-3.86	-5.33	1.24*	-9.80	1.44	2.39	-12.37	-8.78
>7 (n=11)	-5.53	-6.23	3.34*	-10.09	2.60	3.98	-13.91	-9.10
Nº of prior Tx								
1-2 (n=15)	-4.69	-3.14	2.23	-8.78*	2.52	3.57	-11.74	-5.97*
> 2 (n=14)	-6.93	-5.70	2.01	-11.12*	1.18	2.58	-14.43	-11.08*
PSA baseline								
<122 (n=15)	-6.23**	-4.92	2.02	-10.07	1.60	2.87	-12.95	-9.41
≥122 (n=14)	-4.33**	-2.66	2.44	-9.40	2.72	3.80	-13.32	-5.38
LDH baseline								
<454 (n=12)	-6.40	-5.03	1.76	-9.44	1.62	3.21	-12.78	-9.12**
454 (n=12)	-4.49	-2.85	2.44	-9.60	2.64	3.21	-11.80	-6.08**
PA baseline								
<320 (n=14)	-7.28	-5.62**	2.09	-9.98	1.80	3.27	-13.68	-11.20**
≥320 (n=14)	-4.43	-3.24**	2.10	-10.05	1.97	2.94	-12.09	-5.58**

*P≤0.05 according to T-test; **p≤0.05 according to Pearson test

Prognostic value of the CTCs markers.

In addition to the diagnostic value of our CTC-panel, we studied the prognostic impact of these markers to determine their real clinical interest for the management of patients with mCRPC. For that, we defined two groups of patients, those with low or high levels of each marker, using a cutoff defined as the 50, 60 or 70% percentile depending of each marker.

We first investigated the prognostic potential of these CTC markers by Kaplan–Meier survival analyses for PFS and OS. As Table 5.10 shows, high levels of *KLK3, AR, CYP19* and *GDF15* were statistically associated with shorter PFS rates. For OS we found that patients with high levels of *KLK3, AR, GDF15* and *BIRC5* presented poorer survival rates that those with low levels (Figure 5.10). Thus, patients into the group of bad prognosis according to AR-CTC levels presented 16.6 months of OS while the good prognosis group reached a mean OS of 31 months.

Table 5.10. Kaplan-Meier analysis for clinicopathological parameters and CTCs markers				
	Overall survival (OS)		Progression free survival (PFS)	
	mean (95% CI)	p	mean (95% CI)	p value
Performance status				
PS0	31.2 (22.7-39.8)	0.12	8.1 (5.6-10.6)	0.92
PS1/PS2	22.9 (16.5-29.2)		7.6 (5.8-9.4)	
Gleason Score				
≤7	24.4 (17.8-30.6)	0.70	8.7 (6.3-11,1)	0.14
>7	27.7 (17.8-37.5)		6.7 (4.8-8.7)	
Lymph node metastases				
no	**30.7 (23.4-38.1)**	**0.05**	8.7 (6.7-10.7)	0.12
yes	**18.9 (11.4-26.5)**		6.3 (4.3-8.4)	
N° of prior treatments				
≤2	22.03 (13.9-30.1)	0.17	6.5 (4.9-8.2)	0.09
>2	28.4 (22.3-34.6)		9 (6.6-11.4)	
Baseline PSA serum levels				
≤122	25.7 (18.5-32.9)	0.68	7.6 (5.8-9.4)	0.83
>122	24.4 (16.7-32.1)		7.9 (5.4-10.36)	
Baseline LDH levels				
≤320	24.3 (17.3-31.4)	0.67	7 (5.2-8.8)	0.71
>320	22.9 (13.3-32.6)		6.8 (4.6-8.9)	
Baseline PA levels				
≤454	28.4 (22.3-34.6)	0.15	8 (6.5-9.5)	0.84
>454	22.8 (14.4-31.4)		7.7 (5.1-10.9)	
KLK3				
low	**29.65 (23.4-35.9)**	**0.04**	**9.4 (7.2-10.6)**	**0.012**
high	**20.4 (12.8-28)**		**5.9(4.4-7.4)**	
AR				
low	**31 (26.3-35.7)**	**0.002**	**9.3(7.2-11.5)**	**0.002**
high	**16.6 (8.8-24.4)**		**6 (4.3-7.7)**	
CYP19				
low	26.6 (21.3-31.95)	0.12	**8.9 (7,2-10.6)**	**0.015**
high	20.6 (9-32.2)		**5.2 (3-7.3)**	
TUB1				
low	21.5 (16-27)	0.18	8.5 (6.7-10.2)	0.09
high	30 (17.8-31.2)		5.7 (3.2-8.2)	
GDF15				
low	**31.1 (24.9-37.2)**	**<0.001**	**8.6 (6.7-10.3)**	**0.043**
high	**10.6 (6.8-14.5)**		**5.6 (3.5-7.7)**	
BIRC5				
low	**30.5 (23.7-37.3)**	**0.013**	7.7 (6.2-9.3)	0.94
high	**15.8 (9.4-22.2)**		7.7 (4.2-11.2)	
RAB7				
low	19.9 (14.2-25.7)	0.11	8.5 (6.1-11)	0.22
high	30 (21.8-38.3)		6.9 (5.3-8.6)	
SPINK1				
low	23.1 (17.3-28.9)	0.58	8.5 (6.7-10.3)	0.12
high	28.4 (17.3-39.5)		6 (3.7-8.29)	
HR: hazard ratio; CI: confidence interval.				

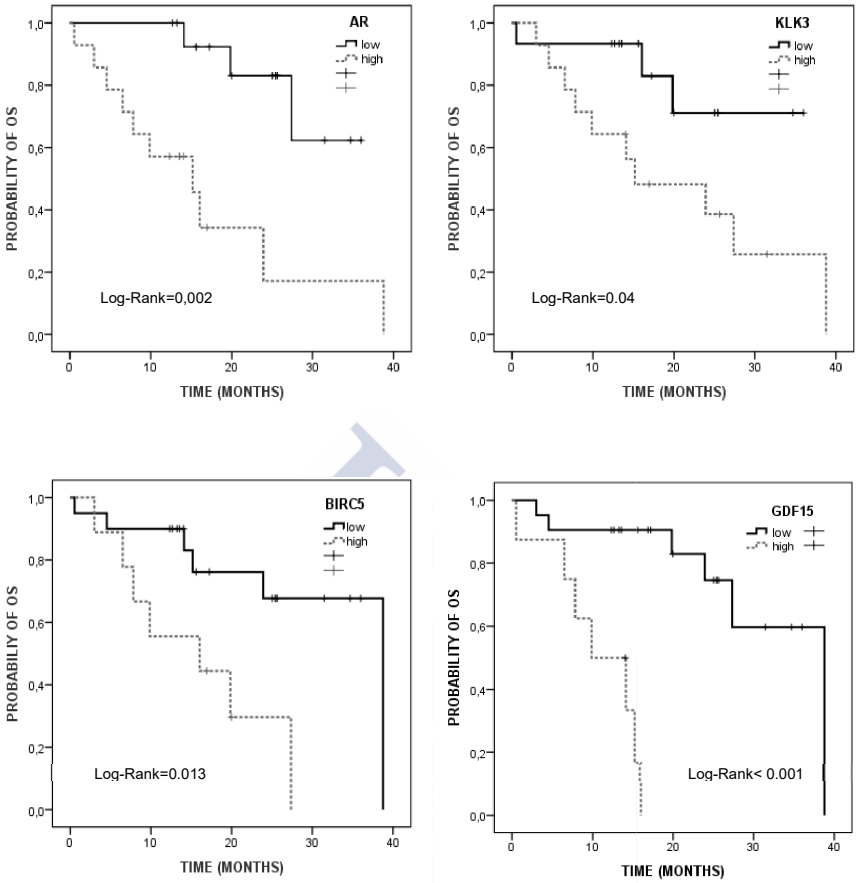

Figure 5.10. Kaplan Meier analysis for overall survival (OS) of validated CTC markers in mCRPC patients. Low/high expression defined based on the 50% (KLK3 and AR) and 70% (BIRC5 and GDF15) percentile.

Univariate Cox regression analysis confirmed the prognosis value of *KLK3*, *AR* and *CYP19* to predict PFS. *AR*, *GDF15* and *BIRC5* were confirmed as good predictors for OS. As Table 5.11 shows, patients with high *GDF15* levels presented a 2.5 and 15.7 fold increased risk of progression and death compared to patients with low *GDF15* levels. It is important to remark that only CTC count and these CTC-markers, among all the other factors including in the study were validated as prognostic markers after the univariate analyses. In fact, *KLK3* (PSA)-CTC associated levels were found as a good prognostic marker for PFS and almost reached statistical significance for OS while

serum PSA levels at baseline did not show any value to predict the response to the chemotherapy and the patient evolution (Table 5.11).

We believe that this panel of markers identified in the population of CTC can provide useful prognostic information in our cohort of mCRPC patients and even play a role as an alternative to CellSearch for detection of CTCs.

Table 5.11. Univariate Cox regression analysis for clinicopathological parameters and CTCs markers

	Progression free survival (PFS)		Overall survival (OS)	
	HR (95% CI)	p value	HR (95% CI)	p value
Performance status (PS0 vs. PS1/PS2)	1 (0.43-2.4)	0.92	4.3 (0.55-34.2)	0.16
Gleason Score (≤7 vs. >7)	1.8 (0.8-4.3)	0.15	0.78 (0.21-2.8)	0.7
Lymph node metastases (no vs. yes)	1.8 (0.83-3.89)	0.13	3.08 (0.91-10.3)	0.06
Nº of prior treatments regimens (≤2 vs >2)	0.5 (0.2-1.15)	0.10	0.44 (0.13-1.5)	0.18
Baseline PSA serum levels (≤122 vs >122)	0.92 (0.42-1.9)	0.83	1.26 (0.4-4)	0.68
Baseline LDH levels (≤320 vs >320)	0.85 (0.37-1.9)	0.7	1.3 (0.39-4.2)	0.67
Baseline FA levels (≤454 vs >454)	0.92 (0.42-2)	0.84	2.4 (0.69-8.6)	0.16
KLK3 (low vs high)	**2.7 (1.2-6.1)**	**0.016**	3.52 (0.94-13)	0.06
AR (low vs high)	**2.5 (1.1-5.58)**	**0.027**	**6.7 (1.7-25.6)**	**0.005**
CYP19 (low vs high)	**2.7 (1.16-6.24)**	**0.020**	2.42 (0.75-7.76)	0.13
TUB1 (low vs high)	2 (0.87-4.8)	0.09	0.36 (0.08-1.7)	0.2
GDF15 (low vs high)	2.4 (1-5.8)	0.05	**15.7 (3.1-79.7)**	**0.001**
BIRC5 (low vs high)	1(0.44-2.35)	0.94	**3.96 (1.2-12.7)**	**0.02**
RAB7 (low vs high)	1.62(0.73-3.6)	0.22	0.39 (0.11-1.3)	0.12
SPINK1 (low vs high)	1.9 (0.8-4.3)	0.13	0.69 (0.18-2.56)	0.58

Hazard ratios, 95% confidence intervals (CI) and p-values values are shown. Statistically significant p-values ($p < 0.05$) are highlighted with bold letters. Marker high and low levels were calculated based on 50, 60 and 70 percentile depending on each marker. *HR: hazard ratio; CI: confidence interval.*

The independent prognostic value of CTCs counts, PSA, LDH and CTCs markers for OS was assessed by multivariate Cox proportional hazards regressions. We included baseline PSA and baseline LDH as clinical variables studied extensively in the literature. We also included the CTCs levels analyzed by CellSearch as the standard for CTCs analyses. For this analysis we only considered those markers with statistically significant p-values in the univariate analysis (we also include *KLK3*, bordering on significance). Comparing different assessment methods, only CTCs counts (HR 1.003; $p = 0.046$) showed independent prognostic significance (Table 5.12).

Table.5.12 Multivariate Cox proportional hazards regression for OS			
	Hazard ratio	95% CI	p
CTC BASAL	1.004	1.001-1.007	**0.018**
PSA basal	0.677	0.143-3.192	0.622
LDH basal	6.583	0.803-53.966	0.079
AR	0.297	0.045-1.937	0.204
KLK3	0.095	0.007-1.283	0.076
BIRC5	0.701	0.163-3.017	0.634
GDF15	0.111	0.012-1.061	0.056

5.4. Global genome expression characterization of CTCs from mCRPC.

CTC Immunoisolation and global gene expression analysis.

The strategy for CTC immunoisolation, RNA extraction and amplification for hybridisation onto cDNA microarrays was previously validated by Barbazan et al for colorectal cancer patients (168). Briefly, CTC were immunoisolated from 7.5 ml of peripheral blood from mCRPC patients (n=9; Table 5.13) at baseline and when patients progressed. Magnetic beads were used, which were coated with a monoclonal antibody towards EpCAM (171). RNA from isolated CTC was purified using a kit specifically designed for low abundance samples. In parallel, the same protocol was applied to blood samples from healthy donors (n=6) to establish the baseline of background from unspecific non-CTC immunoisolation. Prior to gene expression analysis, the presence of isolated CTC was confirmed by CellSearch system quantification and/or the presence of KLK3 transcripts in the CTC fraction by RT-qPCR.

Table 5.13. Clinicopathological parameters of patients analyzed by global gene expression arrays										
Id	Age	ECOG	Gleason score	Lymph node metastasis	Metastasis site	Nº previous treatme nts	CT	Baseline PSA (ng/dl)	Baseline CTCs*	PFS (months)
2	64	1	6	yes	Bone and lung	3	Docetaxel	196	55	7
5	80	1	8	yes	Bone	2	Docetaxel	139	0	9
6	65	1	6	yes	Bone	2	Docetaxel	1.929	59	6
9	52	1	-	no	Bone	3	Docetaxel	409	78	4
11	73	0	8	no	Bone	3	Docetaxel	233	12	10
13	79	1	9	no	Bone	4	Docetaxel	715	12	6
17	71	1	8	no	Bone	2	Docetaxel	3.115	199	9
21	68	1	9	yes	Bone	2	Docetaxel	347	239	11
24	74	1	7	yes	Bone	1	Docetaxel	279	21	6

*analyzed by CellSearch technology; PFS, progression free survival; CT: chemotherapy; Id: identification

In order to characterize the CTC population from mCRPC patients, after the immunoisolation, purified RNA was further treated with DNaseI, amplified using the WTA2 whole transcriptome amplification method, and complementary DNA was labeled and hybridized onto Agilent expression arrays (Gene Expression Omnibus, GEO. Accession number: GSE31023). After the initial pre-processing of raw data, an average of 21,273 spots were filtered according to the criteria described in Materials & Methods, which represented 47.81 % of the spots in the microarray with a maximum of 28,867 (64.88%) and a minimum of 15,629 (35.12%). Normalization among all microarray data was performed by the Quantile method implemented in the Limma package of the R statistical software. This method ensured that the A values (average intensities) had the same empirical distribution across microarrays whilst leaving M values (log-ratios) unchanged.

First, to identify the genes specifically expressed in the CTC population of mCRPC patients from the background of contaminating blood cells isolated together with the CTC population we consider signals obtained in our 6 healthy controls as the background from non-specifically isolated blood cells, mainly lymphocytes. Therefore we consider as non specific CTC genes those genes with positive expression in healthy controls. After discarding the genes expressed in healthy samples, the list of genes specifically expressed in CTCs was composed by those genes present in at least 5 patients at baseline and progression (Table 5.14).

This strategy led to the identification of a final set of 54 genes, 50 of them were annotated genes that were specific to the CTC population in our patients. It is important to remark the presence in this list of *KLK3* (PSA) as the broad accepted prostate cancer marker and *EpCAM*, the molecule used for the isolation of CTCs in our appoach and the one classically used for CTC isolation in carcinomas. In addition, *BIRC5* was also found as member of this list.

On the other hand, we aimed to identify those genes with increased levels at the moment of progression that could be participating in the tumor expansion and the development of resistance to chemotherapy (first-line docetaxel or cabazitaxel). For this purpose we

Table 5.14. Genes specifically expressed in CTCs from mCRPC patients.

TP53TG5	HARBI1
ENST00000372194	TRIM15
XM_001716578	WHAMML1
THBS4	NKAIN4
ROM1	ENST00000412934
MOSPD1	C14orf72
ARL4A	AL050203
QKI	LEAP2
MAOA	PPIL6
BIRC5	DEFA4
FOSL1	FGD4
PAGE2B	ENST00000381747
CREM	**EPCAM**
LOC100130700	NUMBL
A_33_P3215252	LOC286058
KLK3	THPO
ZMYM2	C9orf71
ZBTB49	NBL1
NGEF	CCDC19
TMTC1	SDK1
HOXB13	CCNL2
HOXA3	NLRP4
TULP2	CLTB
ENST00000409758	C18orf25
CADM4	TMCC2
ENST00000409646	GHRL
EFNA1	BIK

analized genes with low expression (lower signal than 361.78=Q1) at baseline and high expression at progression disease (signal higher than 2953.91=Q3). After proccesing the data the list obtained from this analysis was composed by 16 genes and was characterized by the presence of genes such as *CYP3A4* and *CSAG2* strongly associated with resistance to chemotherapy (Table 5.15).

Table 5.15. Genes characterizing CTCs from mCRPC patients at progression.

ACY3	ENST00000404580
AIF1L	OMA1
BC066548	LOC121952
C8orf4	MAGEA6
CNKSR3	LOC100288568
CSAG2	PLAT
CT45A1	TDRD1
CYP3A4	TSPY3

Bioinformatic analysis with Ingenuity Pathway Analysis (IPA).

The analysis of molecular pathways, gene networks and biological functions associated with the list of genes specifically expressed in CTC immunoisolated from mCRPC patients was performed using IPA. Genes expressed in at least five patients and not expressed in any of the controls were considered for IPA analysis. This analysis proposed a number of cellular functions related to the list of 50 CTC genes, all of them highly altered in cancer (Table 5.16).

Table 5.16. Main cellular functions associated with the list of genes specifically expressed in CTC immunoisolated from mCRPC.

CELLULAR FUNCTIONS	GENES
Cell proliferation	*BIRC5, CCNL2, CREM, EFNA1, EPCAM, FOSL1, GHRL, HOXB13, NGEF, THPO, TP53TG5*
Cell death/apoptosis	*BIK, BISC5, CCNL2, CREM, EFNA1, EPCAM, FOSL1, GHRL, HOXA3, HOXB13, KLK3, MAOA, NLRP4, NUMBL, NUPL1, QKI, ROM1, THBS4, THPO, ZMYM2*
Cell cycle progression	*BIRC5, EPCAM, FOSL1, QKI, THPO, ZBTB49, ZMYM2*
Cell differentiation	*ARL4A, BIK, EFNA1, EPCAM, FOSL1, GHRL, XOXB13, KLK3, MINOS1-NBL1/NBL1, NUMBL, QKI, SDK1, THPO, TRIM15, ZMYM2*
Cellular movement/cell invasion	*BIRC5, EFNA1, EPCAM, FGD4, FOSL1, GHRL, HOXB13, KLK3, NUMBL*
Cellular assembly and organization/microtubule dynamics	*BIRC5, EFNA1, FGD4, GHRL, MAOA, MINOS1-NBL1/NBL1, NUMBL, SDK1, THBS4*
Cell morphology/formation of cellular protrusions	*FGD4, EFNA1, GHRL, MINOS1-NBL1/NBL1, NUMBL, SDK1, THBS4*
Cellular attachment/adhesion	*EPCAM, KLK3, EFNA1*
Reproductive system disease/ hypodiploidy of prostate cancer	*BIRC5*
Reproductive system disease/familial prostate cancer	*HOXB13*

Next, we found cell proliferation and cell death together with cell cycle and cellular differentiation as important functions that could illustrate the process CTCs might experience to adapt and survive in the hostile environment of blood stream. Of note, cellular movement and cell invasion was highlighted as a principal biological function defining the prostate cancer-CTC, concordant with the events of CTC homing and extravasation. Moreover, genes associated with microtubule dynamics as main actor during cell migration as well as the formation of cellular protrusions and cell attachment/adhesion, point to an active role of the isolated CTC during the generation of

micrometastasis.

IPA analysis also identified insulin, estrogen receptor 2 (ESR2) and beta-estradiol signaling as upstream regulators of these cellular functions, suggesting these signaling pathways as potential therapeutic approaches specifically targeting the subpopulation of CTC in mCRPC patients. Likewise, the analysis of gene-gene interactions and gene network identified ERK, PI3K/Akt and NOTCH signaling pathways orchestrating the biological features of prostate cancer CTC (Figure 5.11), and therefore are also potential therapeutic targets in these patients.

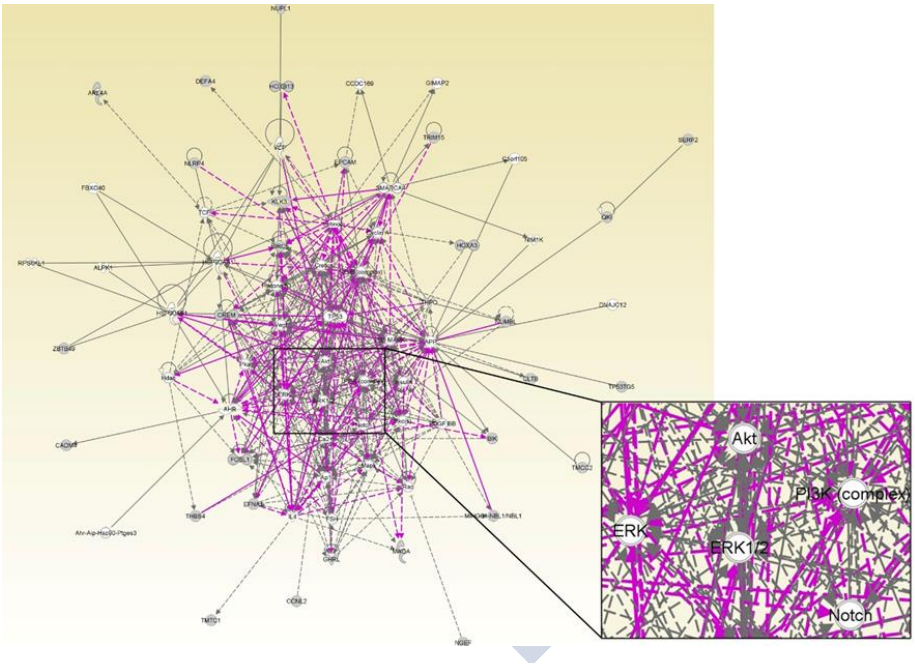

Figure 5.11. Gene network generated with the list of genes specifically expressed in CTC from mCRPC. Signaling pathways at the core of the network are candidate pathways orchestrating the biology of CTCs and potential therapeutic targets.

Regarding the analysis of differential gene expression at disease progression compared to paired samples at baseline from the same patients, we focused on those genes poor expressed at baseline and presenting maximal expression at progression. IPA analysis of the 16 genes obtained with this strategy pointed to apoptosis as the main cellular function related with these genes (*C8orf4, CYP3A4, MAGEA3/MAGEA6, OMA1, PLAT, TDRD1*), indicative of a modulated response to therapy. Gene network analysis pointed

to beta-estradiol as a key molecular actor in the biology of prostate-cancer CTC at progression (Figure 5.12).

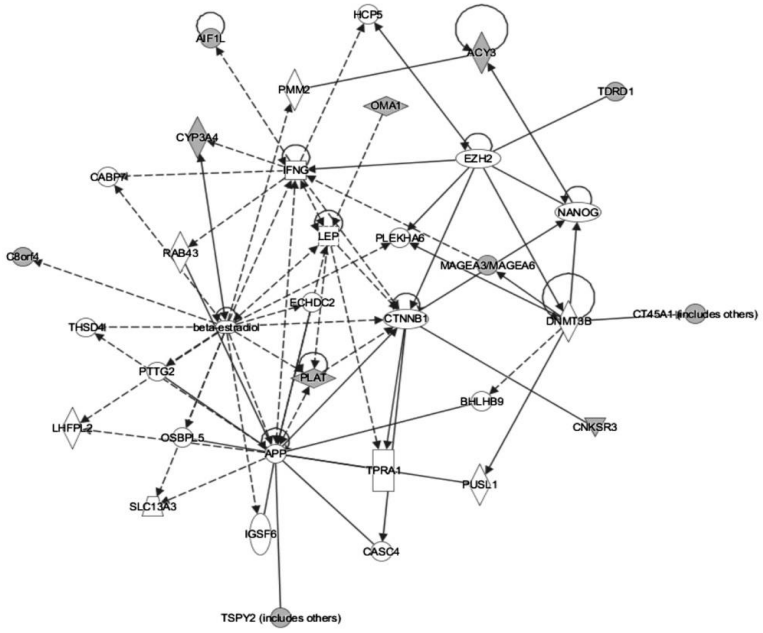

Figure 5.12. Gene network generated with the list of genes specifically expressed in prostate cancer-CTC at progression.

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Chapter 6. Discussion

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Clinical value of CTC counts monitoring for mCRPC management.

Prostate cancer is one of the most common cancers in industrialized countries. Despite the excellent results obtained with surgery or radiotherapy in initial stages, unfortunately 30% of these patients will develop metastatic disease, usually in the bone. Androgen deprivation has been the therapeutic strategy with more effective response, with disease control in 80% of cases, although almost all patients eventually develop resistance to hormone deprivation, a state known as metastatic castration-resistant prostate cancer (mCRPC).

Classically the key elements determining the prognosis and the decision on when to start or finish treatment in mCRPC patients are: clinicopathological features, serum PSA and radiological evaluation (67). Histopathological analysis allows us to know data on the extent of the tumor and other features (such as Gleason score, vascular and lymphatic invasion) that will be useful but do not allow to predict the disease evolution.

Serum PSA is currently used as biomarker to measure disease burden and to monitorize treatment response. One of the disadvantages of PSA is that it can be increased both in PCa as in benign prostatic hyperplasia. Besides, their levels could be similar in indolent and aggressive cancers and often fails to indicate accurately the patient's response to treatment. The PSA flare phenomenon after the onset of chemotherapy in patients with mCRPC may also complicate this situation (172). PSA flare is not related with progression and has not a negative impact in patients outcomes negatively. However, we can not know before 12 weeks if an elevated PSA corresponds to a PSA flare or a real progression (172). For this reason PSA is not accepted for regulatory purposes for the approval of new drugs.

Radiological progression is defined by PCGW2 as the appearance of two or more new lesions in bone scintigraphy (conclusive proof) or progression by RECIST 1.1 criteria (in the case of soft tissue lesions) (64). Limited lymph node and visceral tumor involvement restrict the value of the standard imaging test for evaluation of response. Besides bone metastases are measured with difficulty by bone scans. Although in a recent study Morris et al. have showed a correlation between the radiographic PFS and OS (using a modified form of RECIST 1.0), evaluation of bone disease remains a dilemma in this tumor (173). A high percentage of men experienced bone scan flare in the absence of confirmed bone scan progression over time. This fact complicates

treatment decisions and may lead to the suspension of a treatment that was actually proving effective. Therefore, the clinical application of new surrogate markers will provide the opportunity for improving patient management and the therapeutic selection and monitoring.

During the process of haematogenous spread in PCa, tumor cells travel through the blood vessels and, after extravasation, colonize distant target organs, typically the bone. As circulating tumor cells (CTCs) are consider an intermediate between the primary tumor and metastasis, they are candidates to act as surrogate markers measurable in blood. Taking into account that bone metastases in patients with prostate cancer are difficult to biopsy, CTCs from these patients might serve as a "liquid biopsy" reflecting tumor burden and progression at real time. Moreover, serial monitoring of CTCs could provide information concerning not only tumor burden over time, also about the molecular characteristics of tumour cells evolving under the treatment pressure and being the best tumour source to identify response biomarkers (105).

CTCs occur at very low frequency in the bloodstream, generally estimated at 1 CTC per million of leukocytes. Because of the low concentration of CTCs in blood, extremely sensitive and specific strategies are required to process the blood samples in a short period of time. A considerable number of technologies have been developed to isolate, quantify and characterize CTCs in last years, but only CellSearch platform have been cleared by FDA for clinical use in metastasic breast, colorectal and prostate cancers (124,125,174).

Several studies have established the prognostic value of CTCs for OS in patients with PCa (108,152,154). In the SWOG S0421 study 238 men with mCRPC were treated with docetaxel, and the median OS was 26 months for the group of CTCs<5 versus 13 months for those patients with CTCs≥ 5 per 7.5 mL at baseline. In addition, the group of Bono and collaborators found LDH concentration and CTC counts (better measured as a continuous variable) as the most predictive factor for survival in the IMMC38 trial.

We decided to explore whether the same approach was applied to men with mCPRC treated in our hospital. In this cohort of 29 mCRPC patients the median PFS was 7.4 months and median OS was 27.3. The CTCs count showed prognostic value being the median survival time 16 months for those patients with ≥5 CTCs at baseline versus not reached for those<5 CTCs. This means that only a patient into the favorable CTCs

count died at the end of the follow-up. Importantly, we found higher number of CTCs in patients diagnosed with locally advanced disease, nodal invasion, and also in patients that were responsive to hormonal therapy for less than 24 months, all factors associated with adverse clinicopathological features. But this fact alone justifies the introduction of CellSearch platform in daily clinical practice? Does eliminate CTC count the value of other factors such as PSA or LDH?

Beyond the value of a single baseline measurement kinetics we believe that CTC quantification should also provide predictive information about therapeutic response, and help clinicians with the selection of the most appropriate treatment for each patient at each moment. Thus, drops in CTCs levels within the therapy has been associated with higher OS, similar to the benefit correlated to a substantial PSA decrease or radiographic response (109,110,154,174). Goldkorn et al. described that changes in CTCs levels from day 0 to 21 were prognostic being any increase in CTC counts, as a continuous variable, from day 0 to 21 associated with reduced OS. However, drops in CTC count showed only a trend towards improved OS (154). Authors suggest that perhaps an early drop at day 21 after treatment onset could not be maintained over time and therefore, it makes difficult to know the real prognostic implications of this CTCs reduction, while an early rise after therapy initiation may reflect primary resistance and constitutes a prognostic factor associated with a poor outcome.

Our data are aligned with those described in literature, since patients with CTCs ≥ 5 before the 3rdcycle and 6thcycle had a very high risk of being in biochemical or radiological progression. Another important point which enhances the usefulness of the CTCs counting is that patients who moved from a baseline count ≥ 5 CTC, to CTCs levels <5 after chemotherapy achieved a mean PFS (8 months) and OS (38 months) similar to the group that started with <5 CTC at baseline determination.

Regarding evaluation of response changes in CTCs levels usually precede PSA fluctuation being their monitoring of even greater value when changes in PSA or bone disease are difficult to evaluate (108). Scher *et al.* have described the lack of association between the time to a single rise in PSA and survival (and a second determination improves results) but in contrast a single rise in CTCs was moderately associated to survival time (108). Though still very preliminary, there are examples of the use of CTC in the evaluation of response in patients with prostate cancer. In this sense, Mateo and

collaborators in a study of 50 patients treated with olaparib, considered RECIST 1.1, or PSA level reduction or a confirmed reduction in the CTCs count from ≥5 cells per 7.5 ml of blood to < 5 cells per 7.5 ml for the assesment of response (175).

One limitation of the CellSearch system is the low detection of CTC in mCRPC chemotherapy-naïve patients, a stage where we need to have new intermediates surrogates for OS. Indeed although the survival times of patients with high CTC numbers are uniformly poor, those with favorable counts vary excessively. Therefore we need methods to detect more cells in a higher percentage of patients and help refine the prognosis of patients with favorable cell counts (108).

Another limitation of the Cellsearch platform is that it can only isolate prostate tumor cells expressing EpCAM. Therefore this platform misses those populations of cells that have low or absent EpCAM expression, including those who have undergone epithelial-mesenchymal transition and tumor stem cells. This is of special interest in patients with prostate cancer because androgen deprivation therapy may promote epithelial-mesenchymal transition (176). Furthermore CellSearch does not allow to select cells for *ex vivo* culture or for genomic analysis. Some of these limitations could be overcome by using other methods to detect not only the stem or epithelial-mesenchymal subpopulations but also CTC clusters (177).

To this regard, Ma *et al.* have confirmed in a meta-analysis the strong prognostic value of CTCs (178). They demonstrated that detection of CTCs by immunohistochemistry was less accurate than using CellSearch or RT-PCR. Though only the Cellsearch system has been approved by the FDA, RT-PCR could perform as well as the Cellsearch system in many cases. Notwithstanding, any method can still missed EMT phenotype cells or CTC clusters. However, the complexity and the heterogeneity of the CTC population requires the application of more versatile methods with allow a deeper molecular characterization than CellSearch.

A new approach for CTCs analysis in mCRPC patients based on immunoisolation and RT-qPCR.

Nowadays it is well accepted that CTCs provide a uniquely accessible source of tumor-derived material for molecular analyses, even more important in tumors such PCa where the inaccessible metastatic lesions not allow individualize therapies according to the mechanism of drug resistance, which appear during the evolution of the disease

(179,180). In addition to the CTC enumeration, the molecular characterization of CTCs could provide important insights into disease progression and might allow adaptation of therapeutic strategies, mainly in CRPC patients, since the optimal use of chemotherapy, enzyme inhibitors or AR antagonists require the application of precision molecular medicine.

Our group and others previously demonstated that combination of CTC-immunoenrichment and the analysis of CTC-transcriptome by RT-q-PCR provide an alternative and high sensitivity method for CTCs detection and characterization (131,168,181). Here we use this approach to characterize the CTC population from mCRPC men progressing despite castrate levels of testosterone and after at least one hormonal manipulation. The goal of this study was to identify CTC-markers with clinical impact for the management of these patients starting chemotherapy based on docetaxel or cabazitaxel.

First, we evaluated the expression levels of *GAPDH* normalized to the background of *CD45* expression. *GAPDH* levels were significantly higher in the group of 29 patients compared to controls indicating the presence of an extra population of cells isolated from the blood of CRPC patients. Then we found a CTCs transcriptome phenotype mainly characterized by the expression of two group of genes; those related with androgen signaling pathway such as *AR* and *CYP19* and those implicated in relevant functions for PCa progression and resistance such as *BIRC5*, *TUB1A*, *GDF15*, *RAB7* and *SPINK1.*

Androgen stimulation is essential for prostate gland development and homeostasis and a main actor in PCa evolution. From 2-18% of prostate tumors harbour AR mutations, and 5-52% present AR amplification (182,183). In a recent study in 150 prostate cancer patients, 71% of cases presented AR pathway aberrations, the majority of wich were directed alterations affecting AR through amplification and mutation (183). Nowadays, it is widely accepted that AR pathway is active when patients relapse despite castrate levels of androgens (testosterone and dihydrotestosterone).

The analysis of AR in CTCs was attempted by various groups with promising results (176,184). Miyamoto *et al.* determined the AR signalling status in CTCs from patients under androgen deprivation therapy as a possible indicator for therapy efficacy (132). Reinforcing the value of AR analysis, recent studies proposed the evaluation of AR

modifications in CTCs, including detection of AR-V7 and point mutations as an accessible and valuable tool for treatment selection (163,164,185). Detection of AR-V7 in CTCs from men with metastatic CRPC is not associated with primary resistance to taxane chemotherapy. In AR-V7-positive men, taxanes appear to be more efficacious than enzalutamide or abiraterone therapy, whereas in AR-V7-negative men, taxanes and enzalutamide or abiraterone may have comparable efficacy (164).

Crespo et al have evaluated AR expression in CTC of patients treated with enzalutamide or abiraterone (186). Using the CellSearch platform the authors analyzed AR, CK, and CD45 expression and demonstrated that AR nuclear expression is mantained in CTC at progression to novel endocrine agents. Although it is not currently available, a conjugated antibody for the detection of AR splice variants in the CellSearch platform could be incorporated in the 4th channel to automate the detection of ARV7 in this scenario.

Besides the AR alterations, the overexpression of enzymes responsible for androgen synthesis and metabolism have been also proposed to explain the persistence of hormone-mediating signalling in prostate tumor cells under hormone deprivation state (187). We found that CYP19A1 (Cytochrome P450, Family 19, Subfamily A, Polypeptide 1) was present in the CTC population of our mCRPC patients. This enzyme catalyzes the conversion of androstenedione to estrone, and testosterone to estradiol and has been suggested to be involved in prostate cancer risk and survival by affecting the serum sex hormone milieu (188). Although no previous data exists to this concern, the expression of CYP19A1 in CTCs from patients progressing after androgen deprivation could represent an adaptative mechanism to maintain the hormone stimulation of prostate tumor cells.

On the other hand, we identified *BIRC5, TUB1A, GDF15, RAB7* and *SPINK1* as genes characterizing CTCs of mCRPC patiens. They conform a diverse group of genes with a common role promoting tumor agresiveness and the development of resistance to taxanes-based treatment. BIRC5 or survivin is a member of the inhibitor of apoptosis protein family which mediates tumourigenesis suppressing tumor apoptosis and promoting angiogenesis (189). Its expression in PCa tissues has been related with high Gleason score, chemoresistance and cancer progression (190). Serine Protease Inhibitor Kazal Type 1 (SPINK1) is secreted in the prostate gland and its principal role is the

inhibition of serine proteases such as trypsin. Due to their strong association with ETS rearrangement negative prostate cancers, SPINK1 positive tumors have been proposed as a distinct prostate cancer subtype (191). Its overexpression has also been associated with an increased risk of biochemical recurrence in hormonally and surgically treated prostate cancer cohorts (192). RAB7 is a regulator of intracellular endocytic/membrane trafficking involved in many diseases including prostate cancer and several infectious diseases. In addition to its recognized role in vesicle trafficking, this protein has recently described as a regulator of apoptosis in response to growth factors.

GDF15 (Growth differentiation factor-15), most known as MIC-1 (Macrophage Inhibitory Cytokine1), is a cytokine commonly overexpressed in many cancers, including PCa patients, where serum levels are an independent predictors for overall survival and bone metastasis formation (193). Enhanced level of GDF-15 in prostate tumor cells has been associated with their acquisition of epithelial-mesenchymal transition phenotype and docetaxel resistance, even in prostate cancer stem/progenitor cells (194). Finally, Tubuline alpha 1 (TUBA1A) has not been studied before in PCa, although its upregulation was described in breast cell lines resistant to paclitaxel and in breast cancer patients progressing to taxanes-based therapy (195).

Ideally the study of a panel of genes in the CTC population from mCRPC patients allowed: 1) detect tumor cells by an alternative method to CellSearch; 2) obtain information on the prognosis of patients and 3) better understand the molecular mechanism of the disease, with possible involvement in the selection of therapies. So, in addition to provide more information about the biology of the specific subpopulation of CTC in mCRPC, our gene-expression profiling also permitted the identification of valuable prognostic biomarkers. Thus, high levels of AR, CYP19 and GDF15 were associated with poor PFS rates while AR, GDF15 and BIRC5 were also found as consistent predictors of OS in the univariate analysis. This molecular CTCs-signature could be useful both in the initial diagnosis and as a potential tool to monitor therapy or predict the clinical response. Further analysis in a large study including therapy monitoring should be done to determine the clinical value of these markers.

In this sense different groups have explored the PCR mRNA expression in peripheral blood mononuclear cell fraction and its relationship with systemic disease and tumor load. Danila *et al.* have developed a validation of a prostate cancer-enhanced mRNA

detection assay in whole blood as a prognostic biomarker panel for survival (196). They identified a 5-gene panel consisting of *KLK3, KLK2, HOXB13, GRHL2* and *FOXA1*, and measured these markers in blood samples from 97 CRPC patients with progressive disease. Expression of at least 2 f the 5 genes was a strong predictor for survival, comparable with the CellSearch system.

Dijkstras *et al.* analyzed KLK3, PCA3 and TMPRSS2-ERG mRNA in 20 CPRPC patients (197). KLK3 was detected in 89% of cases, a rate higher than described in other series, and which it is similar to our findings (93%) (198). Although it was a small exploratory study, the authors described that in most patients with positive biomarker expression at baseline a decrease in biomarker expression was detected after three cycles of docetaxel. Marín-Aguilera *et al.* analyzed the molecular profiling of peripheral blood from 43 mCRPC patients. They selected 282 genes through a global gene expression and built a two-gene model (SELENBP1 and MMP9). The combination of the two-gene signature and CTC count showed a strong prognostic significance.

One of the potential limitations of our strategy is that we focus exclusively on the fraction of circulating tumor cells, and we do not analyze the relationship between immune response and survival of our patients. Some groups have explored this relationship. Olmos *et al.* used whole-blood gene profiling to identify gene-expression signatures that stratify CRPC patients with castration-resistant prostate cancer into distinct prognostic groups (199). During the process they managed to build a model with only 9 genes which provided prognostic information. The genes with altered expression in patients with worse prognosis were associated to early erythroid cells and B-cell and T-cell function. On the other hand Ross *et al.* described a six-gene signature (ABL2, SEMA4D, ITGAL, C1QA, TIMP1 and CDKN1A) that separated patients with CPRC into two risk groups: a low-risk group with a median survival more than 34.9 months and a high-risk group with a median survival of 7.8 months (200). The authors concluded that the six-gene model suggests a possible deregulation of the immune system.

In summary our study provide novel insights about the molecular profile of CTCs from mCRPC patients candidates to be treated with taxanes. We identified as specific CTC biomarkers genes highly related with the development of resistance to taxanes and

hormone regulation together with known PCa markers as AR and KLK3. Importantly, these markers showed value to predict the evolution of mCRPC patients and constitute a promising tool for therapy selection and monitoring. Unlike other groups studying panels of genes in peripheral blood without isolation of CTCs in our analysis we use beads coated with EpCAM antibodies to isolate CTCs and then extract the total RNA.

Global genome expression characterization of CTCs from mCRPC.

The interest of CTC population as the principal responsible for PCa dissemination and a valuable source to characterize tumor at real time, has increased exponentially in last years. In fact, changes on CTCs phenotype could reflect tumor evolution under the pressure of systemic therapies, providing a unique opportunity to go insight into the mechanisms regulating prostate cancer biology (177,201).

In our study, after evaluating the CTC counts by CellSearch platform and analyze the genes described by RT-PCR, we performed a global gene expression approach to characterize the CTC population from mCRPC patients in order to identify main actors behind PCa aggressiveness. For that we combined CTC immunoisolation based on EpCAM expression, accurate RNA extraction from very low number of cells, whole-genome amplification and a massive gene-expression profiling for the characterization and interpretation of the biology of CTC in mCRPC patients, as we previously described for colorectal cancer (168). Importantly, we analyzed the CTCs population in nine patients starting docetaxel treatment at baseline and when they progressed with the goal of monitoring possible changes in the CTCs population after therapy pressure and selection.

The profiling approach allowed us the identification of 50 genes by subtracting the background of non-specific isolation of blood cells obtained in a group of healthy controls following the same procedure than patients. Validating our analytical strategy we found KLK3 and EpCAM as components of this list. Both genes are well accepted as specific markers of prostate cancer CTCs but also as molecules implicated in tumor behavior (146,197,202,203). Furthermore, the expression of many of the other CTC-genes has been described above in the prostatic tissue. Most of them are involved in key steps of the prostate carcinogenesis such as PAGE2B (prostate-2B gene associated Protein), MAO (monoamine oxidase) and HOXB13 (homeobox B13) (204-206). Pomerantz et al. has shown that the androgen receptor cistrome undergoes extensive

reprogramming during prostate epithelial transformation in man (207). The marked redistribution of AR binding sites during tumorigenesis represents one of the mos recuerrent epigenetic or genetic alterations yet discovered in prostate cancer. The authors observed that FOXA1, a general pioneer factor, and HOXB13 colocalize at most tumor AR binding sites and they are sufficient to reprogram the AR cistrome. Danila, in the study cited previously, included these two genes within the panel related to prognosis (196).

In our gene list we also found EFNA1, encoding a member of the Ephrin family of membrane receptors involved in cell migration, attachment and spreading that has been described as a potential marker of progression in prostate cancer and BIRC5, also known as Survivin, which has been strongly associated with PCa development, progression, and drug resistance (208-210) .

The genes characterizing CTCs from our cohort of patients are paticipating in mechanisms such as cell proliferation and death, cell cycle and cellular differentiation, and cell migration and adhesion. These fuctions are consistent with a subpopulation of tumor cells that must acquire an aggressive and invasive phenotype allowing dissociation from the primary tumour, invasion of neighbour tissues and their intravasation and survival in the blood flow. For example, the activity of BIRC5 in CTCs could be important to prevent the mechanisms of cellular death induced by a high hostile environment such blood, since this molecule have a key role to inhibit apoptosis. In fact, it is known that regulation of apoptosis has a central role in the development of prostate cancer and its progression to an androgen-independent state, which is due, in part, to up-regulation of antiapoptotic genes such as Survivin (211,212).

The challenges associated with CTC detection and analyses begin with the natural scarcity of CTCs therefore platforms for CTC detection with high sensitivity, specificity, and reliability are in need. One area of intense development is the single-cell analysis of CTC. Chen et al. have developed a method for the analysis of single CTC (213). They described that many EpCAM-positive CTCs show loss of epithelial characteristics. These cells may not express PSA an other markers related with primary prostate tumors. Interestingly these cells become highly deformable by increasing elasticities of their membrane. In other work by Miyamoto et al established single-cell RNA-sequencing profiles of 77 intact CTCs isolated from 13 patients by using

microfluidic enrichment (214). Single CTCs from each individual display considerable heterogeneity, including expression of AR gene mutations and splicing variants. CTCs from patients progressing under an AR inhibitor, compared with untreated cases, indicates activation of noncanonical Wnt signaling.

In our study we performed an Ingenuity Pathway analyses of the list of genes specifically expressed in CTC immunoisolated from mCRPC patients. Genes expressed in at least five patients and not expressed in any of the controls were considered for IPA analysis. Interestingly, our CTC profiling before docetaxel treatment pointed out ERK, PI3K/Akt, NOTCH and insulin as relevant cell signaling pathways for CTC biology, all of them classical tumor driver pathways in cancer. We also found ESR2 and beta-estradiol pathways as relevant for CTC biology in our cohort of mCRPC patients. In this sense, estrogens dependent regulation of prostate cancer was described as an important mechanism promoting tumor progression when cancer cells become androgen independent (215). This effect may be related to: 1) estrogens interference with PI3K-Akt signaling pathway, ultimately resulting in apoptosis inhibition and an increase in cell cycle progression, and 2) up-regulation of insulin-like growth factor receptor by 17B-estradiol, with increased mitogenic and motogenic activities in response to IGF (216). Therefore, our CTC-profiling could provide a realistic picture of the main determinants in an androgen independent status.

On the other hand, comparing the CTC gene expression profile before docetaxel and when the patients progressed we found a small number of differentially expressed genes. In this list CSAG2 and CYP3A4 stand out as regulators of chemotherapy activity (217). CYP3A4 encodes a member of the cytochrome P450 superfamily of enzymes that catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids. It is nowadays established that cytochrome P450-3A is a major determinant of the variability in docetaxel response being its inhibition a broad accepted strategy to improve docetaxel efectiveness in prostate cancer and other tumors (218,219). The higher presence of CYP3A4 transcripts in CTC population of mCRPC patients could reflect the expansion of a more resistant tumour population after docetaxel pressure.

Finally, it is important to note that CTCs study has also been used to guide treatment selection in clinical trials. In breast cancer, HER2 protein expression on CTCs has been

assessed using the CellSearch technology, and other investigators are using CellSearch to monitor endocrine resistance in ER-positive HER2-negative metastatic disease (COMETI phase II trial, NCT01701050).

In prostate cancer various studies explore clinical applications of CTCs. CTCs baseline number will enable better risk stratification of patients at the time of inclusion in clinical trials. In addition one of the secondary objectives in the ongoing trial NCT02379390 is to analyze tumoral mRNA including AR-V7 as a biomarker in CTCs, an information with direct involvement in treatment selection. Another study (NCT02485691) evaluates the correlation of a signature of resistance to AR-targeted agents with clinical outcome via the analysis of CTC phenotypes as well as expression and localization of proteins including AR isoforms in CTCs. A similar strategy will implement the CTC analysis to select which patients will benefit from treatment (and will continue to) and which patients are in progress and should switch to a new agent.

Overall, our global expression analysis described a specific molecular profiling of CTC isolated from mCRPC within docetaxel treatment. This approach allowed us to get a better picture of the principal actors for prostate cancer progression after androgen deprivation. We found a general stress-survival phenotype in the CTC population of mCRPC patients partially based on cell proliferation, apoptosis, adhesion and migration. Importantly we identify relevant therapeutic drivers when patients progressed that could represent potential monitoring markers and therapeutic targets after docetaxel treatment. Our results could be usefullness to improve the clinical management of of mCRPC patients but a deeper validation should be attempted in a bigger independent cohort. First, to determine the robustness of DNA microarrays we should perform a RT-qPCR of the selected group of 15 genes. The next step will be to develop a clinical trial in patients with mCRPC with serial blood samples to confirm the value of our panel of genes in the detection, prognosis and response evaluation. But that is another story.

Chapter 7. Conclusions

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1. In our cohort of 29 mCRPC patients starting chemotherapy CTCs levels analyzed using CellSerach showed prognostic value. The number of CTCs was higher in patients diagnosed with locally advanced disease, nodal invasion, and also in patients that were responsive to hormonal therapy for less than 24 months.
2. CTCs count showed value as a therapy monitoring marker. Thus, patients with CTCs ≥ 5 before the 3rdcycle and 6thcycle of chemotherapy had a very high risk of being in biochemical or radiological progression. On the contrary, man who moved from a baseline count ≥ 5 CTC, to CTCs levels <5 after chemotherapy achieved similar results to the baseline low risk group.
3. Combination of CTC-immunoenrichment and the analysis of CTC-transcriptome by RT-qPCR provide an alternative and high sensitivity method for CTCs detection and characterization in mCRPC patients.
4. *KLK3* together with those genes related with androgen signaling pathway (such as *AR* and *CYP19)* and those implicated in relevant functions for PCa progression and resistance (such as *BIRC5*, *TUB1A*, *GDF15*, *RAB7* and *SPINK1)* are specific CTCs markers.
5. High levels of *AR, CYP19* and *GDF15* were associated with poor PFS rates while *AR, GDF15* and *BIRC5* were also found as consistent predictors of OS.
6. Besides the prognosis value of the CTCs-signature identify by RT-qPCR this markers could be useful both in the initial diagnosis and as a potential tool to monitor therapy or predict the clinical response.
7. The validity of our method combining CTCs immunoisolation based on EpCAM expression, accurate RNA extraction from very low number of cells, whole-genome amplification and a massive gene-expression profiling for the characterization and interpretation of the biology of CTCs in mCRPC patients has been shown.
8. A specific set of 50 genes was found to characterize the population of CTCs from our cohort of patients. These genes are paticipating in relevant mechanisms for cancer progression such as cell proliferation and death, cell cycle and cellular differentiation, and cell migration and adhesion.

9. After a bioinformatic analysis the CTCs profiling before docetaxel treatment pointed out ERK, PI3K/Akt, NOTCH and insulin as relevant cell signaling pathways for CTC biology, all of them classical tumor driver pathways in cancer. We also found ESR2 and beta-estradiol pathways as relevant for CTC biology in our cohort of mCRPC patients.
10. The comparison of the CTCs phenotype between baseline and when patientes progressed provided us a list of genes, such as CYP3A4, that could be related with a more chemoresistant tumor subpopulation.

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Resumen

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EL CÁNCER DE PRÓSTATA.

El cáncer de próstata es el segundo tumor con mayor mortalidad en hombres de paises industrializados. A pesar de que generalmente presenta un pronóstico favorable existe un grupo de pacientes que desarrollan metástasis a distancia, habitualmente a nivel óseo, y que finalmente fallecen como consecuencia de esta enfermedad (1).

El diagnóstico de cáncer de próstata se basa en tres elementos: *prostate specific antigen* (PSA) elevado, tacto rectal sospechoso y biopsia de próstata. La determinación de PSA en sangre ha jugado un papel fundamental en el aumento de detección de cáncer de próstata en estadios iniciales visto en las últimas décadas. El riesgo de cáncer de próstata aumenta con valores elevados de PSA, pero no existe un umbral de PSA por debajo del cual se pueda descartar definitivamente que no existe riesgo de padecer un tumor maligno prostático.

El sistema de estadificación se basa en el AJCC TNM, pero también tiene en cuenta el PSA sérico y Gleason. La enfermedad localizada se debe clasificar en grupos pronósticos para la toma de decisiones (12). Siguiendo un modelo predictivo se puede determinar la probabilidad de control bioquímico a los 5 años después del tratamiento local (90%, 60% y 30%, respectivamente):

- Bajo riesgo: PSA <10 ng/dL, Gleason ≤ 6 y T1c o T2a.
- Riesgo intermedio: PSA 10-20 ng/dL, Gleason 7 y T2b.
- Alto riesgo: PSA> 20 ng/dL, Gleason ≥ 8 y T2c.

Los pacientes con grado de Gleason elevado tienen mayor probabilidad de invasión linfovascular, márgenes quirúrgicos positivos, mayor riesgo de extensión extraprostática y de aparición de metástasis. A pesar de su utilidad y de las mejoras introducidas en las revisiones de 2005 y 2014, el sistema de clasificación de Gleason todavía tiene importantes limitaciones. Epstein *et al.* han propuesto una adaptación con posibles beneficios: una estratificación más precisa que los sistemas actuales y una simplificación a cinco grados, con el potencial de reducir el sobretratamiento (10).

La mayoría de los pacientes son diagnosticados con tumores confinados a la próstata. Las opciones de tratamiento varían según la edad, el grado de Gleason, comorbilidad, expectativa de vida y otras características clínicas. En el cáncer de próstata de bajo riesgo, el paciente debe decidir entre cirugía o radioterapia frente a la vigilancia activa.

La vigilancia activa es apropiada para muchos pacientes, especialmente hombres con tumores menos agresivos y edad avanzada.

En el cáncer de próstata de riesgo intermedio se puede plantear prostatectomía radical con linfadenectomía extendida o radioterapia externa asociada a tratamiento hormonal adyuvante. La braquiterapia es también una alternativa en pacientes seleccionados.

En aquellos pacientes con enfermedad de alto riesgo muchas veces es necesario plantear un tratamiento multimodal, idealmente decidido dentro de un comité multidisciplinar. Las alternativas son la prostatectomía radical con linfadenectomía ampliada (y radioterapia adyuvante si se precisa tras el análisis de la pieza quirúrgica) o la administración de radioterapia externa acompañada de 2-3 años de tratamiento hormonal.

A pesar de los excelentes resultados obtenidos con cirugía o radioterapia, desafortunadamente un 30% de estos pacientes desarrollarán enfermedad metastásica. Historicamente la deprivación androgénica ha sido la estrategia terapéutica con una respuesta más efectiva, con mejoría y control de la enfermedad en el 80% de los casos, aunque practicamente todos los pacientes acabarán desarrollando resistencia a la deprivación hormonal (220). En pacientes asintomáticos se puede considerar la opción de una segunda maniobra hormonal aunque no está demostrado su beneficio en téminos de supervivencia. En pacientes con bloqueo hormonal completo y que presentan signos evidentes de progresión la retirada de antiandrógeno consigue respuesta en el 20 o 30% de los pacientes.

En 2004, la combinación de docetaxel y prednisona demostró una mejoría en la supervivencia global en hombres con cáncer de próstata metastásico resistente a la castración (mCPRC) (50,51). Recientemente se han comunicado los datos de dos ensayos clínicos aleatorizados que anticipan el uso de docetaxel a los pacientes con enfermedad hormono sensible (62,63) .

Desde los ensayos iniciales de Tannock y Petrylak abiraterona (AA), enzalutamida (ENZ), cabazitaxel, Radium-223 y sipuleucel-T han demostrado prolongar la supervivencia global en pacientes con mCPRC post docetaxel (69,73,80,81,86). Además, AA y ENZ también han logrado beneficio en supervivencia global en pacientes mínimamente sintomáticos quimio-naive, algo que sólo sipuleucel-T-había demostrado previamente (70,75,81). Sin embargo, el 10-20% de los pacientes son

refractarios primarios a AA o ENZ, de ahí que una caracterización adecuada de los pacientes evitaría retrasos indeseables de los tratamientos potencialmente más eficaces.

Tradicionalmente, la evaluación de la respuesta a los agentes terapéuticos en el cáncer de próstata ha sido particularmente complicada debido a la ausencia de lesiones fácilmente medibles, a la prevalencia de la diseminación ósea y la historia natural relativamente prolongada en un número significativo de pacientes.

Hasta la fecha, los niveles de PSA han sido el biomarcador más utilizado para evaluar la progresión tumoral en pacientes con cáncer de próstata. Sin embargo, en muchos casos los niveles en sangre de este marcador no reflejan fielmente el estado de progresión de la enfermedad o el riesgo de aparición de nuevas metástasis. En ocasiones no se consigue un manejo adecuado de la enfermedad debido a la falta de recursos precisos para su detección temprana o para evaluar la respuesta tumoral. Evitar la continuación de un tratamiento inefectivo posibilita reducir la toxicidad del mismo y que el paciente se pueda beneficiar de una nueva estrategia terapéutica.

CÉLULAS TUMORALES CIRCULANTES EN CÁNCER DE PRÓSTATA.

La diseminación temprana de células tumorales en pacientes con cáncer es generalmente indetectable mediante análisis histopatológico convencional o mediante técnicas de imagen de alta resolución. Esta circunstancia ha motivado el desarrollo de ensayos inmunocitoquímicos y moleculares que permiten la detección específica de células tumorales metastáticas en ganglios linfáticos regionales, sangre periférica o médula ósea, antes de que la metástasis tenga el tamaño suficiente para ser detectable macroscópicamente. La detección precoz de células tumorales diseminadas en linfa y médula ósea (DTC) o sangre periférica (CTC) presenta un gran valor para la identificación de pacientes que precisen tratamiento sistémico tras el abordaje local del tumor primario (221).

Asworth en 1869 describió la importancia del estudio de las células tumorales circulantes, pero hasta hace pocos años no existía la tecnología adecuada para su identificación y aislamiento. Actualmente la detección de CTCs se puede realizar de modo directo o indirecto. La detección directa incluye métodos citométricos, en los que se detectan células completas, y en la detección indirecta se utilizan métodos basados en la expresión de genes específicos de este tipo de células. Para intentar mejorar la sensibilidad de estas técnicas de detección, se han desarrollado métodos de

enriquecimiento celular mediante filtración, gradientes de densidad o aislamiento inmunomagnético.

Actualmente el único sistema de detección y análisis de CTC aceptado para uso clínico por la *Food and Drug Administration* (FDA) y en distintos países europeos es el CellSearch™ (Veridex). Este sistema realiza un inmunoaislamiento de células epiteliales con partículas magnéticas unidas a anticuerpos anti-EpCAM (antígeno superficie de célula epitelial altamente expresado en carcinomas). Posteriormente estas células son identificadas por expresión positiva de las citoqueratinas 8 y 18 (marcadores de célula epitelial), expresión negativa de CD45 (marcador de leucocitos) y marcaje positivo para DAPI para demostrar la integridad nuclear y, por tanto, celular. Este sistema proporciona un método altamente reproducible y sensible para la identificación de CTCs (125,222,223) .

En varios estudios se ha relacionado el descenso de niveles de CTC con una mejor supervivencia global, de modo similar al beneficio relacionado con una disminución marcada de PSA o una respuesta radiológica (107,109,110,154). Además los cambios en los niveles de CTC habitualmente preceden las fluctuaciones de PSA y su monitorización es incluso de mayor valor cuando los cambios en el PSA o la enfermedad ósea son difíciles de evaluar (108). Con todo y a pesar del prometedor valor clínico de las CTCs en pacientes con cáncer de próstata, todavía existe una importante necesidad de mejorar los métodos de análisis, que permitan una caracterización molecular adecuada de dichas células y mejoren la sensibilidad y coste-efectividad del CellSearch. Además del valor de la cuantificación de CTCs, el estudio de perfiles moleculares en pacientes con mCPRC puede permitir identificar marcadores con valor clínico en el manejo de estos pacientes.

En nuestro planteamiento, combinamos el aislamiento de CTC basado en EpCAM y el análisis posterior con RT-qPCR de un panel de genes implicados en la vía de señalización mediada por andrógenos, en el fenotipo tumoral *stem like*, resistencia a drogas y en un comportamiento tumoral más agresivo. Con este enfoque, previamente validado en cáncer colorrectal y endometrial por nuestro grupo (168,181), pretendemos identificar un panel de biomarcadores que nos permita identificar las CTC de pacientes con mCPRC, que aporte información pronóstica en supervivencia libre de progresión y

supervivencia global, y que nos proporcione información sobre las características moleculares de dichas células.

HIPÓTESIS DEL ESTUDIO.

Hasta la fecha, los niveles de PSA han sido el biomarcador más utilizado para evaluar la progresión en pacientes con cáncer de próstata. Sin embargo, en muchos casos, los niveles en sangre de PSA no reflejan con precisión el estado de la enfermedad o el riesgo de desarrollo de metástasis. Además, la evaluación radiológica es difícil. Por lo tanto, el tratamiento eficaz de la enfermedad metastásica requiere herramientas clínicas para seleccionar y monitorizar el tratamiento.

Teniendo en cuenta este escenario, hay dos prioridades para mejorar el manejo clínico de los hombres con CPRC metastásico: la caracterización de biomarcadores pronósticos, de seguimiento y respuesta a la terapia para guiar la intervención clínica y la identificación de nuevas dianas terapéuticas mejorando las opciones terapéuticas de estos pacientes.

Debido a la continua evolución de los tumores, que implica la alteración genética y epigenética celular y la heterogeneidad del tumor, se acepta que los tumores primarios y las metástasis individuales proporcionan una información limitada sobre el estado molecular del cáncer. En este sentido, las CTCs proporcionan en tiempo real y secuencialmente una " biopsia líquida " para pacientes con cáncer metastásico. Estas células pueden proporcionar información fundamental para profundizar en la comprensión de la biología del tumor y en los mecanismos de diseminación tumoral.

Nuestra hipótesis es que, además del valor clínico del recuento de CTC para predecir la evolución del paciente con mCPRC, la caracterización molecular de su población de CTC ofrece una fuente única para obtener información importante sobre:

1) El pronóstico de los pacientes.

2) La monitorización de los pacientes para poder determinar una respuesta precoz, anticipando y mejorando la evaluación bioquímica y radiológica.

3) La selección del tratamiento, identificando mecanismos de resistencia a las terapias actuales.

4) Desarrollo del tratamiento, proporcionando información sobre los principales factores implicados en la progresión y agresividad del cáncer de próstata resistente a la castración.

OBJETIVOS DEL ESTUDIO.

Pretendemos explorar las posibilidades de uso de las CTCs en el manejo de pacientes con cáncer de próstata metastásico resistente a la castración. Hemos establecido tres objetivos principales:

1. La evaluación y cuantificación de CTCs en una cohorte de pacientes con CRPC metastático y tratados con docetaxel o cabazitaxel. Evaluar el papel del recuento de CTCs como un marcador independiente de supervivencia libre de progresión y la supervivencia global en este grupo de pacientes. Además, el muestreo secuencial a lo largo del tratamiento nos permitirá evaluar la utilidad de la CTCs como un marcador precoz de la respuesta o resistencia a la quimioterapia.
2. El desarrollo de una metodología más sensible y versátil que constituya una alternativa al sistema de referencia para el análisis de CTCs. Esta metodología se basa en el análisis de un conjunto de marcadores expresados en la población de CTCs de los pacientes en la cohorte del estudio, con el fin de encontrar nuevos marcadores para el diagnóstico y pronóstico que guien la terapia en estos pacientes.
3. La caracterización molecular de las CTCs presentes en pacientes con mCPRC usando un enfoque global de la expresión génica con el fin de identificar nuevos marcadores de CTCs específicos y genes potencialemente relacionados con la resistencia a la quimioterapia.

AISLAMIENTO DE CÉLULAS TUMORALES CIRCULANTES.

En primer lugar realizamos la evaluación y cuantificación de CTCs en una cohorte de 29 pacientes con cáncer de próstata resistente a la castración metastásico, tratados con quimioterapia. La detección de CTC en sangre periférica se correlacionó con datos clínicos y patológicos, y se calculó el porcentaje de respuestas por PSA, imagen, supervivencia libre de progresión y supervivencia global. Se extrajeron 7,5 ml de sangre por paciente en cada muestra, y el recuento de CTC se realizó con el sistema Cellsearch, antes del 1^{er,} 3er y 6^{o} ciclo de tratamiento (docetaxel o cabazitaxel).

La supervivencia libre de progresión (SLP) fue 7,4 meses (IC 95%, 5,9–8,7) (Figura 5.1, apartado “results”) y la supervivencia global (SG) 27,3 meses (IC 95%, 16–38.7) (Figura 5.2, apartado “results”). Se demostró respuesta por PSA en el 55,2% de los pacientes; en el subrupo de hombres con enfermedad medible no se observó ninguna

respuesta radiológica, y en un 48% de los participantes sí se consiguió estabilización del tumor.

El numero de CTCs fue mayor en los pacientes diagnosticados con enfermedad localmente avanzada (28 vs 263; p = 0,054), con invasión ganglionar (33 vs 443; p = 0,03), y que respondieron a tratamiento hormonal durante menos de 24 meses (18 vs 311; p = 0,04). La superviencia global media fue 16 meses (IC 95 % 9,4-24,7) para los pacientes con ≥ 5 CTCs en la extracción basal versus no alcanzada para aquellos con <5 CTCs .

En el análisis mediante curvas ROC, el recuento basal de CTC fue el factor de riesgo con mayor AUC para predecir la posibilidad de tener una supervivencia global por encima de la media (AUC = 0,784). Los pacientes con ≥ 5CTC al inicio del estudio tenían un mayor riesgo de progresión a las 12 semanas (36%, frente al 10% para los hombres con < 5CTCs) y una SLP y SG menor que aquellos con < 5CTC. Sin embargo los pacientes con conversión de ≥ 5CTC a < 5CTC después de 3 ciclos de docetaxel tuvieron una supervivencia libre de progresión y supervivencia global similares a aquellos pacientes con niveles basales bajos de CTC. Nuestros hallazgos concuerdan con los datos publicados por otros autores, describiendo que los pacientes que tienen más de 5 CTC/7,5 mL de sangre tienen un peor pronóstico. De hecho este umbral se utiliza de modo generalizado para establecer el pronóstico de los pacientes con cáncer de próstata metastásico (grupo favorable y otro desfavorable) tratados tanto con quimioterapia como con otros agentes (107,108,152-154).

Resulta especialmente importante el hecho de que los pacientes con ≥ 5CTC en el momento basal y que pasan a < 5CTC durante el tratamiento tienen una supervivencia global similar a los pacientes de buen pronóstico inicial. La respuesta por CTC puede servir entonces no solo como factor pronóstico antes del inicio de tratamiento, si no también para comprobar la eficacia del mismo. En el ensayo SWOG S0421 se estudió la respuesta por CTCs 21 días tras el inicio de docetaxel (con o sin atrasentan). Mientras que el descenso de CTCs se asociaba a una tendencia en mejor supervivencia, pero sin alcanzar la significación estadística, cualquier aumento en los niveles de CTC sí se relacionó con peor pronóstico (154). Puede que los cambios precoces en CTC no discriminen una respuesta prolongada, mientras que su aumento indique pacientes refractarios primarios al tratamiento y con muy mal pronóstico.

En nuestro estudio hemos podido comprobar que la extracción y análisis de muestras de sangre para recuento de CTCs en nuestro medio es factible, consiguiendo resultados similares a otros grupos. Hemos observado una relación entre el número de CTCs en la determinación basal y la supervivencia global y también hemos comprobado el valor pronóstico de la conversión de ≥ 5CTC a < 5CTC tras la adminsitración de quimioterapia.

CARACTERIZACIÓN DE CTC EN mCRPC.

Paralelamente en la primera extracción se recogió un tubo adicional de sangre para llevar a cabo la caracterización molecular de las CTCs presentes en estos pacientes. Las muestras recogidas para la caracterización molecular se procesaron mediante el sistema CELLection Epithelial Enrich, que se basa en el empleo de partículas magnéticas acopladas a anticuerpos anti-EpCAM para la purificación de CTCs. Estas muestras enriquecidas en CTC se utilizaron para extraer mRNA mediante un proceso optimizado para muestras con escasa carga celular junto con un proceso de preamplificación previo.

La presencia de células inespecíficas en la muestra hizo necesario un proceso de selección para asegurar que realmente estábamos analizando el componente celular epitelial. Con esta finalidad primero evaluamos los niveles de expresión de GADPH como un marcador de celularidad, que incluye tanto las CTCs como células de la serie blanca, normalizado por la expresión de CD45 como un marcador específico de células de origen hematopoyético (168).

Tras el inmunoaislamiento analizamos la fracción enriquecida de la muestra mediante q-RT-PCR para el estudio de genes relevantes relacionados con regulación androgénica (*AR, CYP19* and *CYP17*), fenotipo stem cell (*CD133, CD44, ALDH1A, ABCG2, CD49f*) y agresividad de cáncer de próstata y/o resistencia a taxanos (*BIRC5, CLU, GDF15, RAB7A, SPINK1, TUB1A, MDR1*). Se compararon los resultados de los 29 pacientes con un grupo control de 15 hombres sin diagnóstico de cáncer. Los niveles de *GAPDH* fueron mayores en el grupo de pacientes, confirmando la existencia de una población celular "extra" aislada en la sangre de estos pacientes. Además no se detectó expresión de *KLK3* en los controles y sí en el 93,1 % de los pacientes, reforzando nuestra estrategia de selección y análisis de CTCs.

De los genes estudiados observamos una expresión significativa de *AR*, *CYP19*, *BIRC5*, *TUB1A*, *GDF15*, *RAB7* and *SPINK,* un panel que caracteriza la población de CTCs de

nuestros pacientes. Además de su significado diagnóstico exploramos posteriormente su valor pronóstico mediante análisis de Kaplan Meier para SLP y SG. Así, niveles elevados de *KLK3, AR, CYP19* y *GDF15* se asociaron de un modo estadísticamente significativo a peor SLP. Respecto a la supervivencia global los pacientes con niveles elevados de *KLK3, AR, GDF15* y *BIRC5* presentaron una supervivencia más corta que aquellos con niveles bajos. Por ejemplo, los pacientes de mal pronóstico según los niveles de AR-CTC presentaron una superviencia de 16.6 meses mientras que los del grupo de buen pronóstico alcanzaron una media de 31 meses.

El análisis univariante de Cox confirmó el valor pronóstico de *KLK3, AR, CYP19* y *GDF15* para predecir PFS, mientras que solo *AR, GDF15* y *BIRC5* mostraron valor como predictores para supervivencia global. Posteriormente introdujimos estos marcadores en el análisis multivariante para SG, en el que también incluímos niveles basales de CTC analizados mediante CellSearch, PSA y LDH. Únicamente los niveles de CTC alcanzaron la significación estadística.

En una tercera etapa se completó el estudio de expresión génica mediante *microarrays*, siguiendo el protocolo desarrollado en nuestro grupo de investigación (168). La caracterización molecular de las CTC presentes en pacientes con mCPRC se realizó en 9 pacientes y 6 sujetos sanos. Todas las muestra para el análisis de expresión se sometieron a un proceso de amplificación y los datos obtenidos tras este análisis comparativo entre los grupos establecidos se evaluaron a través de herramientas bioinformáticas (análisis de rutas funcionales y redes de genes a través de Ingenuity Pathway Analysis, IPA).

Tras descartar los genes expresados en las controles sanos, la lista de los genes específicos de las CTCs estaba compuesta por los genes presentes en al menos 5 pacientes al inicio del estudio y a la progresión. Esta estrategia condujo a la identificación de un conjunto final de 50 genes específicos de la población CTCs en nuestros pacientes. En este listado se encontraban *KLK3* y *BIRC5,* ambos descritos previamente como marcadores CTCs en nuestra cohorte de 29 pacientes mCPRC cuyas CTCs se analizaron por RT-qPCR (ver sección 5.3). Estos resultados validan nuestra estrategia analítica para caracterizar la población de CTCs en mCRPC.

Por otro lado, identificamos aquellos genes con niveles aumentados en el momento de la progresión y que podrían participar tanto en el crecimiento tumoral como en el

desarrollo de resistencia al tratamiento (docetaxel o cabazitaxel). Identificamos 16 genes, entre los que destacan CYP3A4 y CSAG2, fuertemente asociados con la resistencia a la quimioterapia.

En un último paso analizamos mediante Ingenuity Pathway Analysis (IPA) las vías moleculares, las redes de genes y las funciones biológicas asociadas con la lista de genes expresados específicamente en CTC de nuestros pacientes con mCRPC. Interesantemente nuestro perfil de CTC antes del tratamiento con docetaxel señaló a ERK, PI3K / Akt, Notch y a la insulina como vías de señalización celular relevante para la biología de las CTCs, todas ellas vías clásicas relacionadas con cáncer. También ESR2 y la vía de beta-estradiol resultaron elementos relevantes en la biología de las CTCs de esta cohorte de pacientes.

En resumen, el análisis de expresión global describió un perfil molecular específico de CTCs aisladas en mCPRC en tratamiento con quimioterapia. Este enfoque nos ha permitido profundizar en los principales actores de la progresión del cáncer de próstata tras deprivación androgénica. Encontramos un fenotipo de una población de CTCs basado parcialmente en la proliferación celular, apoptosis, adhesión y migración. Estas vías relevantes en la progresión tumoral podrían representar marcadores de monitorización y potenciales dianas terapéuticas tras tratamiento con taxanos. Nuestros resultados podrían ser de utilidad para mejorar el manejo clínico de estos pacientes, aunque deben validarse un cohorte independiente de pacientes mCPRC con mayor tamaño muestral.

CONCLUSIONES.

1. En esta cohorte de 29 pacientes con mCRPC el recuento de CTC mostró valor pronóstico siendo la mediana de supervivencia de 16 meses para los pacientes con ≥ 5 CTC al inicio del estudio y no alcanzada en los pacinetes con < 5 CTC.
2. Encontramos un mayor número de CTCs en pacientes diagnosticados con enfermedad localmente avanzada, invasión ganglionar, y también en pacientes que eran sensibles a la terapia hormonal durante menos de 24 meses, todos elos factores relacionados con características clínico-patológicas adversas.
3. Los pacientes con CTC ≥ 5 antes del 3er y 6º ciclo de la quimioterapia tuvieron un alto riesgo de progresión bioquímica o radiológica. Por el contrario, los pacientes que pasaron de un recuento basal ≥ 5 CTC a niveles de CTC <5

después de docetaxel o cabazitaxel lograron una SLP media y SG similares al grupo con <5 CTC en la determinación inicial.

4. La combinación de enriquecimiento de CTCs y el análisis del transcriptoma de CTCs mediante RT-qPCR constituye un método alternativo y de alta sensibilidad para la detección y caracterización de las CTCs.
5. Es importante destacar que cuando se comparó la expresión de KLK3, como un marcador específico de células prostáticas, no se encontraron casos positivos en el grupo de controles, mientras que el 93,1% de los pacientes fueron positivos. Este hecho refuerza la alta especificidad de nuestra estrategia para la detección y análisis de CTCs. La curva ROC mostró una alta sensibilidad y especificidad de KLK3 para detectar la presencia de CTCs en nuestra cohorte de pacientes mCRPC.
6. El transcriptoma de las CTCs se caracteriza principalmente por la expresión de dos grupo de genes; los relacionados con la vía de señalización de andrógenos como (AR y CYP19) y los implicados en las funciones relevantes para la progresión y la resistencia del cáncer de próstata, como BIRC5, TUB1A, GDF15, RAB7 y SPINK1.
7. Niveles altos de AR, CYP19 y GDF15 se asociaron con malos resultados de SLP mientras que AR, GDF15 y BIRC5 se mostraron como predictores consistentes de SG en el análisis univariado. Este firma molecular de las CTCs- podría ser útil en el diagnóstico inicial y como una herramienta potencial para monitorizar el tratamiento o predecir la respuesta clínica.
8. Se ha demostrado la validez de nuestro método que combina el inmunoaislamiento de CTCs basado en la expresión de EpCAM, la extracción de RNA de muy bajo número de células, la amplificación de todo el genoma y un análisis global de perfil de expresión de genes para la caracterización e interpretación de la biología de las CTCs en pacientes mCRPC.
9. Se ha descrito un conjunto específico de 50 genes para caracterizar la población de las CTCs de nuestra cohorte de pacientes. Estos genes participan en mecanismos tales como la proliferación y la muerte celular, el ciclo celular y la diferenciación celular, y la migración y adhesión celular.
10. En el análisis IPA el perfil de CTCs antes del tratamiento con docetaxel señaló a

ERK, PI3K / Akt, Notch y la insulina como vías de señalización celular relevantes para la biología de las CTCs, todas ellas vías clásicas en cáncer. También ESR2 y la vías de beta-estradiol son elementos relevantes en la biología de las CTCs en nuestra cohorte de pacientes.

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List of abbreviations

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3D-CRT: 3-dimensional conformal radiation therapy.

AA: abiraterone acetate.

ADT:androgen deprivation therapy.

AE: adverse effect.

AR: Androgen receptor.

AR-V7: AR splice variant 7.

BFS: biochemical-free survival.

CAB: combined androgen blockade.

CBC: Cancer Biomarkers Collaborative.

CCP: cell cycle progression.

CI: Confidence interval.

CNV: copy number variations.

COPD: Cronic Obstructive Pulmonary Disease.

CRPC: castration resistan prostate cancer.

CSS: prostate cancer-specific survival.

CT: computed tomography.

CTC: circulating tumor cells.

CV: coefficient of variation.

DAPI: 4,6-diamidino-2-phenylindole.

DRE: Digital rectal examination.

DTC: disseminated tumor cell.

EBRT: external beam radiation therapy.

ECOG: Eastern Cooperative Oncology Group.

EGAPP: Evaluation of Genomic Applications in Practice and Prevention.

EpCAM:epithelial cell adhesion molecule.

ESR2: estrogen receptor 2.

FDA: US Food and Drug Administration.

HE: hematoxylin eosin.

IMRT: Intensed Modulated radiotherapy.

IPA: Ingenuity pathway analysis.

MFS: metastasis-free survival.

MRI: magnetic resonance imaging.

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NCCN: National Comprehensive Cancer Network

NED: Neuroendocrine differentiation.

NYR: not yet reached.

LH-RH: luteinizing hormone-releasing hormone.

OS: overall survival.

PCa: prostate cancer

PCWG: Prostate-Specific Antigen Working Group.

PFS: progression free survival.

PLND: pelvic lymph node dissection.

PPV: Predictive positive value.

PSA: Prostatic specific antigen.

RANKL: Receptor Activator of Nuclear Factor Kappa B Ligand.

RECIST: The Response Evaluation Criteria in Solid Tumours.

REMARK: Reporting Recommendations for tumor MARKER prognostic studies.

ROC: Receiver Operating Characteristic.

RP: radical prostatectomy.

rPFS: radiographic progression-free survival.

SBRT: Stereotactic body radiotherapy.

SOGUG: Spanish Oncology Genitourinary Group.

SRE: skeletal related events.

TURP: Transurethral resection of the prostate.

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Annex

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Annex 1. Clinical Database.

A. Age.
B. Comorbidity.
    a. Hypertension.
    b. Chronic obstructive pulmonary disease.
    c. Diabetes mellitus.
    d. Heart disease.
    e. More than one of the previous.
    f. Other diseases.
    g. None of these.
C. Initial treatment.
    a. Date of diagnosis.
    b. Initial stage.
    c. PSA.
    d. Gleason.
    e. Initial treatment.
        i. Surgery (yes/no). Date of surgery.
        ii. Radiotherapy (yes/no). Date of radiotherapy. Indication (adyuvant or radical). Median dose.
D. Treatment of the metastatic disease, previous to chemotherapy.
    a. Hormonal treatment.
        i. Number and date of hormonal treatments.
        ii. Kind of maneuver:
            1. Complete blockade.
            2. Antiandrogen.
            3. LH-RH agonist.
            4. Antiandrogen withdrawal.
            5. Ketoconazol.
            6. Flutamide.
            7. Others.
        iii. Reason to hormonal treatment stop.
            1. Imaging progression.
            2. PSA progression.
            3. Clinical PSA.
            4. Progression by two factors.
            5. Progression by three factors.
            6. Toxicity.
            7. Others.
    b. Biphosphonates (yes/no).
    c. Radionuclide (yes/no).
    d. Paliative radiotherapy (yes/no).

E. Treatment of the metastatic disease at the start of chemotherapy.
    a. Stage: TNM.
    b. LDH, Alkaline phosphatase, PSA.
    c. Chemotherapy.

i. Docetaxel or cabazitaxel.
ii. Date of start and finish.
iii. Total dose. Number of cycles.
iv. Reduction (yes/no). Delay (yes/no).
v. Toxicity: adverse event and grade.

F. Evaluation of response.
   a. Clinical response.
   b. Biochemical response.
   c. Radiological response.

G. Second-line treatment.
   a. Docetaxel.
   b. Mitoxantrone.
   c. Ciclofosfamide.
   d. Vinorelbine.
   e. Paclitaxel.
   f. Abiraterone.
   g. Cabazitaxel.
   h. Clinical trial.
   i. Others.
   j. None.

H. Disease progression.
   a. Progression (yes/no).
   b. Date of progression.
   c. Site of progression:
      - Loco-regional.
      - Bone.
      - Lung.
      - Mediastinal.
      - Liver.
      - CNS.
      - Others.
      - More than one site.

I. Survival.
   a. Exitus (yes/no).
   b. Date of death.
   c. Cause of death.
      i. Progression disease.
      ii. Toxicity.
      iii. Infection.
      iv. Other.

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Annex 2. Informed consent.

HOJA DE INFORMACIÓN PARA EL PARTICIPANTE

Análisis de tejido tumoral y sangre

Código de Participante: _____

Título del estudio: "Estudio de las células tumorales circulantes de pacientes con cáncer de próstata resistente a deprivación androgénica" Código de Protocolo: CTC-PROST-01
Promotor: Dr. Rafael López López. CHU de Santiago de Compostela.

Nombre del Investigador: Tel.: .....
Dirección: .....

¿En qué consiste este estudio? ¿Cuáles son sus objetivos?
El Servicio de Oncología Médica de este Hospital está llevando a cabo un estudio para entender mejor los mecanismos que permiten que el cáncer de próstata progrese formando metástasis en otros órganos diferentes al de origen y que deje de responder a fármacos que se emplean para combatirlo, como el Docetaxel. Para ello se necesita aislar y estudiar las células tumorales que se encuentran en sangre de pacientes como usted.

Para que las células del tumor se asienten en otros órganos, como por ejemplo los huesos, tienen que viajar por la sangre, donde pueden ser detectadas. Estas células tumorales detectadas en sangre también se denominan células tumorales circulantes o CTC. Se ha observado que el número y las características de las CTC varían una vez iniciado un tratamiento con quimioterapia. Estudiar estos cambios es de gran importancia para encontrar nuevas herramientas para seguir la evolución de su enfermedad y para comprender mejor el funcionamiento del cáncer de próstata y, por tanto, encontrar nuevas vías de tratamiento. Es por ello, por lo que solicitamos su consentimiento para la utilización de muestras de su sangre para hacer nuestro estudio.

Este estudio ha sido aprobado por el Comité Ético de Investigación Clínica y se realizará siguiendo la Declaración de Helsinki y los requisitos establecidos en el Real Decreto 223/2004.

¿Cómo se realizará este estudio?

Solicitamos su consentimiento para que durante la visita previa al primer tratamiento con Docetaxel se le extraigan dos tubos adicionales de sangre. Una de estas muestras de sangre se utilizará para estudiar características de estas células tumorales circulantes en sangre que se asocien a la resistencia al tratamiento o a la capacidad de formar metástasis en distintos órganos. La otra muestra servirá para ver los niveles de células tumorales circulantes presentes en su sangre.

Estos niveles de células tumorales circulantes se cuantificarán además en una muestra que se le extraerá antes del 3er y 6º ciclo de tratamiento.

En el momento en que consideremos que su tumor se ha vuelto resistente al tratamiento le solicitaremos 1 tubo adicional de sangre para estudiar características del tumor que se asocien a la resistencia al tratamiento.

Tenga en cuenta que estas muestras son un valioso instrumento para la investigación, que podría permitir obtener información para nuevas terapias y estrategias para pacientes, que como usted, padecen esta misma enfermedad.

Es importante que sepa que su médico dentro del estudio podrá informarle de los resultados de los análisis, si usted así lo desea.

¿Cuáles son los beneficios esperables y los riesgos potenciales de este estudio?

Usted no obtendrá ningún beneficio por permitir el análisis de sus muestras biológicas. En cualquier caso, la información que se obtenga por su participación en este estudio puede ser de gran ayuda para otras personas con su misma enfermedad. La cesión de muestras para investigación es voluntaria y altruista. Su único beneficio es el que corresponde al avance de la medicina en beneficio de la sociedad, y el saber que ha colaborado en este proceso.

Las muestras no podrá ser objeto directo de actividades con ánimo de lucro. No obstante, la información generada a partir de los estudios realizados sobre su muestra podría ser fuente de beneficios comerciales. En tal caso, están previstos mecanismos para que estos beneficios reviertan en la salud de la población, aunque no de forma individual en el donante.

Su participación en este estudio es completamente voluntaria. Si usted decide no participar recibirá todos los cuidados médicos que pudiera necesitar y su relación con los equipos médicos que le atiendan no se verá afectada. La participación en este estudio no tendrá ningún coste para usted.

Las únicas molestias y riesgos de participar en este estudio están relacionados con la toma de la muestra de sangre de su brazo, que se realiza siguiendo el procedimiento habitual. Le puede ocasionar un pequeño hematoma o una leve inflamación, que desaparecerán en pocos días; más raramente, mareo o dolor en el momento de la extracción de sangre.

¿Se dispone de otros tratamientos?

En esta parte del estudio que ahora se le propone no se pretende evaluar ningún tratamiento adicional sino que simplemente queremos estudiar como afecta el tratamiento con Docetaxel a la población de células tumorales que circulan a través de su sangre y las posibles causas de resistencia a este fármaco.

Su participación es voluntaria:

Si desea participar en esta parte del estudio debe comunicárselo a su médico del estudio. Su participación es voluntaria. Si interviene en este estudio, debe saber que en cualquier momento puede decidir no seguir participando sin tener que manifestar razón alguna para ello.

¿Qué sucederá con las muestras?

La muestra de sangre fresca que se extraiga será almacenada en el Servicio de Oncología. Si usted nos da su consentimiento, dichas muestras serán trasladas al Laboratorio de Oncología Traslacional del Hospital Clínico Universitario de Santiago, para su posterior análisis. Durante este tiempo, dichas muestras se codificarán con su número de participación en el estudio, pero nunca con su nombre y apellidos o con cualquier otro dato que pueda identificarlo de manera directa.

Las muestras se conservarán en el Laboratorio de Oncología Traslacional del Hospital Clínico Universitario de Santiago durante como máximo 5 años. Pasado este tiempo, las muestras de sangre serán destruidas. Ninguna de las muestras recogidas se utilizará para otras investigaciones, ni serán compartidas con ningún otro equipo de investigación.

Revisión de Documentos Originales, Confidencialidad y Protección de Datos de Carácter Personal:

Usted comprende y consiente: con el fin de garantizar la fiabilidad de los datos recogidos en este estudio, será preciso que los miembros del equipo investigador, así como el promotor del estudio y eventualmente las autoridades sanitarias y/o miembros del Comité Ético de Investigación Clínica, tengan acceso a su historia clínica, comprometiéndose a la más estricta

confidencialidad, de acuerdo con la Ley 41/2002. Así mismo, se dará cumplimiento a la Ley de Investigación Biomédica 14/2007.

El tratamiento, la comunicación y la cesión de los datos de carácter personal de todos los sujetos participantes se ajustará a lo dispuesto en la Ley Orgánica 15/1999, de 13 de diciembre de protección e datos de carácter personal y la transmisión de datos se hará conforme a la dicha ley y al R.D. 1720/2007. De acuerdo a lo que establece la legislación mencionada, usted puede ejercer los derechos de acceso, modificación, oposición y cancelación de datos, para lo cual deberá dirigirse a su médico del estudio. En ninguno de los informes del estudio aparecerá su nombre y su identidad no será revelada a persona alguna salvo para cumplir con los fines del estudio y en el caso de urgencia médica o requerimiento legal. Cualquier información de carácter personal que pueda ser identificable será conservada y procesada por medios informáticos bajo condiciones de seguridad por el Promotor, o por una institución designada por el, con el propósito de determinar los resultados del estudio. El acceso a dicha información quedará restringido al personal del estudio designado al efecto o a otro personal autorizado que estará obligado a mantener la confidencialidad de la información. Los resultados del estudio podrán ser comunicados a las autoridades sanitarias y, eventualmente, a la comunidad científica a través de congresos y/o publicaciones.

Sus datos se transferirán de forma codificada, no incluyendo iniciales, nombre, dirección u otro dato que le identifique directamente. Le será asignado un número que sólo el equipo médico del estudio podrá conectar con su nombre. De acuerdo con la ley vigente, usted tiene derecho al acceso de sus datos personales; asimismo, tiene derecho a su rectificación y cancelación. Si así lo desea, deberá solicitarlo al médico que le atiende en este estudio.

Otra información que usted debe conocer:

Los resultados del estudio podrán ser comunicados en reuniones científicas, congresos médicos o publicaciones científicas. Siempre se mantendrá una estricta confidencialidad sobre su identidad.

Si usted lo desea, su médico de cabecera será informado de su participación en este estudio, para lo cual se le entrega a usted una carta informativa para que se la haga llegar.

Ante cualquier eventualidad que pudiera surgir mientras participe en este estudio o para cualquier pregunta sobre el mismo que desee realizar tras leer este documento, por favor diríjase a: _____, en el teléfono _____.

Se le entregará copia de este documento firmado y fechado.

Este documento se firmará por duplicado quedándose una copia el Investigador y otra el Participante

CONSENTIMIENTO INFORMADO
Análisis de CTC en sangre

Yo, (nombre y apellidos)_____

He leído la hoja de información que se me ha entregado.
He podido hacer preguntas sobre el estudio.
He recibido suficiente información sobre el estudio.

He hablado con: (nombre del investigador) _____

He tenido tiempo suficiente para considerar de manera adecuada mi participación en el estudio.
Comprendo que mi participación es voluntaria.
Comprendo que puedo retirarme del estudio:

1. Cuando quiera
2. Sin tener que dar explicaciones
3. Sin que esto repercuta en mis cuidados médicos

Presto libremente mi conformidad para que se utilicen mis muestras y datos asociados como parte de este proyecto de investigación.

Además:

Afirmo haber obtenido información adecuada sobre la finalidad de la conservación, el lugar de la conservación, la seguridad y las garantías de cumplimiento de la legalidad vigente que me proporciona el centro encargado de conservar las muestras a los fines indicados tal efecto.

Comprendo que sobre las muestras de este estudio NO se llevarán a cabo análisis genéticos.

Autorizo a que se transfieran mis muestras y datos asociados, excepto los que me identifiquen, al Laboratorio de Oncología Traslacional del Hospital Clínico Universitario de Santiago.

Fecha: ……. /……. /……. Fecha: ……. /……. /…….

Firma del Investigador Firma del Participante

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DE COMPOSTELA

Annex 3. Ethical research committee approval.

XUNTA DE GALICIA
CONSELLERÍA DE SANIDADE
Secretaría Xeral

Comité Ético de Investigación Clínica de Galicia
Edificio Administrativo de San Lázaro
15781 SANTIAGO DE COMPOSTELA
Tlf: 881 546425 Fax: 881 541804
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galicia

DICTAMEN DEL COMITÉ ÉTICO DE INVESTIGACIÓN CLÍNICA DE GALICIA

Paula M. López Vázquez, Secretaria del Comité Ético de Investigación Clínica de Galicia

CERTIFICA:

Que este Comité evaluó en su reunión del día 15/12/2011 el estudio:

Título: Estudio de las células tumorales circulantes de pacientes con cáncer de próstata resistente a deprivación androgénica
Promotor: Luis León Mateos
Version:
Código do Promotor: CTC-PROST-01
Código de Registro CEIC de Galicia: 2011/408

Y que este Comité de conformidad con sus Procedimientos Normalizados de Trabajo y tomando en cuenta los requisitos éticos, metodológicos y legales exigibles a los estudios de investigación con seres humanos, sus muestras o registros, emite un **DICTAMEN FAVORABLE** al estudio propuesto y que se llevará a cabo en:

Centros	Investigadores principales
C.H.Universitario de Santiago	Luis León Mateos

En Santiago de Compostela a 20 de diciembre de 2011
La Secretaria

Paula M. López Vázquez

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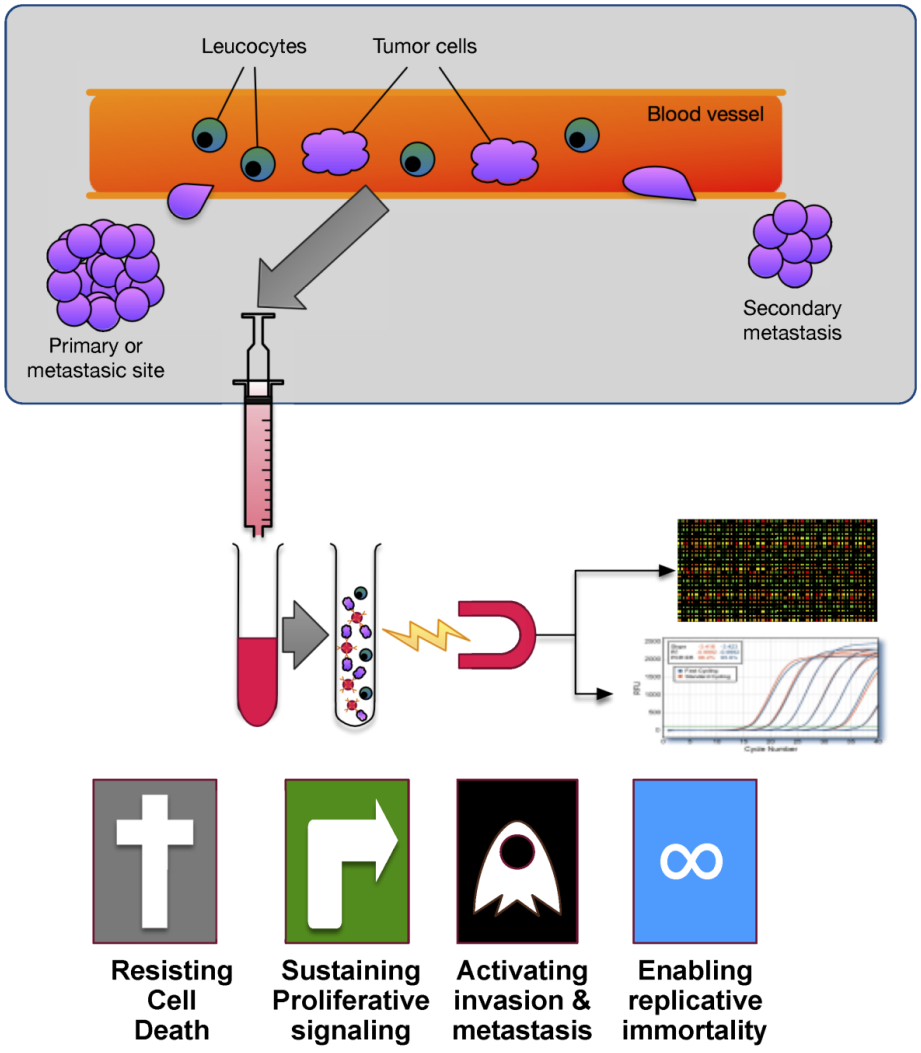
Annex 4. Graphical Abstract.



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Agradecimientos.

Gracias a los pacientes y a sus familias. Su generosidad y colaboración en este estudio han sido absolutas. La ilusión por contribuir a profundizar en el conocimiento de su enfermedad, y la dignidad con la que se comportan en momentos tan difíciles merece toda nuestra admiración y respeto.

Del mismo modo nuestro trabajo no habría sido posible sin el apoyo, paciencia y cariño de todo el personal del Hospital de Día y de la planta de Oncología del Hospital Clínico Universitario de Santiago. Para todos y cada uno de sus miembros, mi más sincero agradecimiento.

Como persona y como médico he sido educado en el valor que aporta el equipo y el trabajo compartido. Resulta imposible, a pesar de la visión cortoplazista y gremial en la que en ocasiones nos escudamos, no desarrollar nuestro trabajo dentro de un enfoque interdisciplinar, en colaboración con otros compañeros. De ahí que el mérito del presente trabajo sea compartido también con miembros de otros servicios del hospital: Anatomía Patológica, Unidad de Farmacia Oncológica, Medicina Nuclear, Oncología Radioterápica, Radiodiagnóstico o Urología, entre otros.

En el laboratorio de Oncología traslacional del Hospital Clínico se realizaron los estudios de enumeración y caracterización molecular de las células tumorales circulantes. Lógicamente sin su participación no existiría esta tesis. Por su disponibilidad, conocimiento y capacidad inagotable de respuesta muchas gracias, en especial a Helena, Alicia, Miguel y Laura.

María aportó siempre una visión original y disruptiva desde Canadá, además de batir el record mundial de número de variables en una base de datos. A Urbano le debo varias ilustraciones magníficas, una valiosísima ayuda estadística, y la revisión de parte del texto. Además Urbano ha participado en el cuidado de muchos de los pacientes que nos han cedido sus datos, y hemos compartido ideas, inquietudes. Hemos aprendido juntos y siempre ha estado a mi lado.

Rafa e Ihab, mis directores de tesis, han encontrado tiempo (ese bien tan preciado) para poder dedicármelo y guiarme tanto en el diseño inicial del estudio como aconsejarme en el enfoque final del manuscrito. Han contribuido con sus sugerencias a la revisión del texto.

Gracias también a Miguel por su contribución a la parte de arrays, y por la revisión de la discusión y conclusiones. Es especialmente motivador el que siempre esté receptivo a cualquier propuesta, y nos anime a seguir desarrollando proyectos.

Gracias a Fernando, por su ánimo y por el cuadro que ilustra la portada y el inicio de cada capítulo.

Antonio y Roberto también me han regalado parte de su tiempo, además de proporcionarme excelentes fotografías incorporadas en la introducción. Más allá y mucho más importante, comparto con ellos una profunda y estimulante amistad.

Por su profunda y exhaustiva revisión del manuscrito, por su paciencia, por su claridad y extenso conocimiento del mundo de las CTCs estoy en completa e impagable deuda con Laura. Ahora que deposito la tesis ganará mucho en calidad de vida.

El ejemplo de mi padre y mi madre, su dedicación, profesionalidad y valores han sido siempre y siguen siendo un referente en mi vida. Es una gran suerte poder darles las gracias a mis padres...y seguro que se sorprenden, porque les parecerá que no han hecho nada. Solo ser padres. Todo.

Javi, Sabela, Miguel y Pedro han sufrido directamente estos meses, el tiempo insuficiente que les he dedicado. A cambio solo he tenido sonrisas, alegría y palabras de ánimo. Ilusión. Para vosotros, por vuestra paciencia, por vuestra curiosidad insaciable, por transmitir tanta felicidad.

A mis hermanos y al resto de mi familia.

Bea me animó desde antes del primer momento, siendo consciente que sería la principal damnificada en este proceso. Ha sonreído en el cansancio, el estrés, los momentos de duda, y en las dificultades. Me ha cuidado en todo momento. Ha participado también de la emoción, del progreso del texto, y ha acogido este proyecto como común. Ha esperado pacientemente y ha aprendido todo lo que no debe hacerse en el desarrollo de la tesis.

Gracias por arriesgar, Bea, y por creer en nuestra vida juntos.